Biochimica et Biophysica Acta 1848 (2015) 105–114

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

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

Valeria Capurro [a], Ambra Gianotti [a], Emanuela Caci [a], Roberto Ravazzolo [a,b], Luis J.V. Galietta [a], Olga Zegarra-Moran [a,*]

[a] Istituto Giannina Gaslini, Via G. Gaslini 5, Genova I-16148, Italy
[b] Dipartimento di Neuroscienze, Riabilitazione, Oftalmologia, Genetica e Scienze Materno Infantili, Università di Genova, Italy

ARTICLE INFO

Article history:
Received 8 July 2014
Received in revised form 26 September 2014
Accepted 2 October 2014
Available online 13 October 2014

Keywords:
Patch-clamp
pH
Phosphatidylinositide-3-kinase
Tyrosine-protein kinase
Dynamin
DIDS

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

* Corresponding author at: U.O.C di Genetica Medica, Istituto Giannina Gaslini, Via G. Gaslini 5, Genova I-16148, Italy. Tel.: +39 010 56362403; fax: +39 010 3779797.
E-mail address: ozegarra@unige.it (O. Zegarra-Moran).

http://dx.doi.org/10.1016/j.bbamem.2014.10.008
0005-2736/

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

estimating the shift in the current reversal potential when 125 mM Cl was replaced by equimolar concentration of different anions.

The conductance to voltage relationship was obtained. To this end, the current was divided by the delta V_m, as defined by the Goldman–Hodgkin–Katz equation of the form

$$\text{delta}V_m = V\frac{(1-\exp[(-V-E_{rev})F/RT])}{1-\exp(-VF/RT)} \quad (2)$$

where V is the applied membrane pulse potential, E_{rev} is the reversal potential, F is the Faraday's constant, R is the universal gas constant and T is the absolute temperature. The activation curve was fitted with a Boltzmann function of the form:

$$G/Gm = \frac{1}{1+\exp[(Vm-Vh)zF/RT]} \quad (3)$$

where Vh is the membrane potential at which the conductance is half maximal and z is the gating charge associated with voltage-dependent channel opening. Tail currents were obtained at membrane potentials between −100 and 60 mV after voltage jumps from a pre-pulse to 100 mV. The instantaneous value of the tail current was measured fitting the current decay with an exponential function and extrapolating to the beginning of the test pulse.

2.3. Transfection procedure

HEK-293 cells were transfected using Lipofectamine 2000 following the manufacturer's instructions. Cells were transfected with pools of three (TMEM16 family) or four (ClC family) sequences of specific siRNA at a concentration of 10 nM each. As a negative control were used non-targeting sequences.

2.4. Real time PCR (RT-PCR)

After silencing ClC-3, ClC-7, TMEM16A-D-F-H and K, the detection of corresponding mRNA levels was assessed in HEK cells. Total RNA was extracted using both Tryzol (Gibco–BRL) and RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. One microgram of spectrophotometer-quantified RNA was retro-transcribed using IScript RT Kit (Biorad). RT-qPCR was carried out using inventoried Assays-on-DemandTM provided by Applied Biosystems (Hs00923164_m1 for ClC-3, Hs01126462_m1 for ClC-7, Hs00400770_m1 for TMEM16D, Hs01374899_m1 for TMEM16F, Hs00326325_m1 for TMEM16H, Hs00216307_m1 for TMEM16K and Hs00187842_m1) for the gene encoding β2-microglobulin (used as endogenous control). RT-PCR was performed using IQ5 real-time PCR Detection System (BioRad). Cycling conditions were as follows: 3 min hot start at 95 °C followed by 40 cycles of denaturation steps at 95 °C for 30 s, and annealing and extension steps at 60 °C for 30 s. Each sample was run in triplicate. Changes in mRNA amount were quantified by using the comparative CT Method (Sequence Detection System Chemistry Guide, Applied Biosystems). The same method was used to detect the expression levels of these genes in the different cell lines.

2.5. Materials

SiRNA oligonucleotides against TMEM16A, TMEM16D, TMEM16F and TMEM16H were from Life Technologies Italia (Monza, Italy). SiRNA oligonucleotide against TMEM16K was from Sigma-Aldrich. SiRNA oligonucleotides complementary to ClC-3 and ClC-7 were the Smart-pools from Dharmacon (Euroclone, Milan, Italy) consisting in four siRNA sequences against the same target gene. All other chemicals were purchased from Sigma-Aldrich (Milan, Italy).

2.6. Statistical analysis

Results are presented as raw data or as means ± SEM. Unpaired Student t test was used to analyse results and differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Current response to acidic extracellular pH

The application of an acidic bath solution (pH 4.65) to HEK-293 cells stimulated by alternating pulses to 100 mV and to −100 mV, respectively, caused a rapid (<4 second) increase of outward and inward currents (Fig. 1A). While at positive potentials the current rapidly reached a constant value and remained stable as far as the acidic solution was present, the current increase at −100 mV was transient. The addition of 100 μM amiloride blocked the inward without altering the amplitude of outward current. This result suggests that the transient response is due to the activation of the acid-sensing ion channels (ASIC) and that activation of ASIC is not necessary for the activation of the current at positive potentials. The substitution of extracellular gluconate for Cl^- completely abolished the outward current response to pH 4.65, suggesting that it was carried by Cl^- ions. Therefore, hereafter we will call this current acid or proton-activated Cl^- current, $I_{Cl(H)}$. We analysed the current response to a reduction of bath pH to 4.65 in different cell lines, including primary human bronchial epithelial cells (HBE), neuroblastoma SK-N-MC cells, RAW264.7 macrophages, three cell lines that derived from cystic fibrosis patients (pancreatic adenocarcinoma CFPAC and bronchial epithelia lines CuFi and CFBE41o-), bronchiolar H441 cells, thyroid FRT cells, ovary CHO and human embryo kidney HEK-293 cells. All cells responded to pH 4.65 with a rapid increase of $I_{Cl(H)}$ and displayed similar kinetics. The current density measured at 100 mV was largely homogenous and close to 100 pA/pF with the exception of CHO cells that expressed higher current levels (Fig. 1B).

3.2. pH dependence of $I_{Cl(H)}$

A side-by-side comparison of the characteristics of $I_{Cl(H)}$ from different cell types in the same conditions has never been reported. This study analysed the pH dependency of $I_{Cl(H)}$ in primary HBE cells, SK-N-MC cells and HEK-293. The three cell lines responded to pH 5.4 with a slight current increase. A more marked increase was obtained when pH was further lowered to pH 5 or 4.65 (see the traces and current to voltage relationships for HBE cells in Fig. 1C and D). The currents were strongly outwardly rectifying. Normalized current to pH relationships were fitted with a Hill equation giving an apparent EC_{50} at pH values of 5.16, 5.15 and 5.14, and a Hill coefficient n_H of 2.93, 3.54 and 3.23 for SK-N-MC, HBE and HEK-293 cells, respectively. The EC_{50} value did not change with V_m. However, n_H increased from 1.6 at −100 mV to 4.1 at 100 mV (inset to Fig. 1E).

3.3. Anionic permeability and voltage dependence of $I_{Cl(H)}$ channels

The reversal potential of $I_{Cl(H)}$, measured at pH 4.65 in HBE and HEK-293 cells, was 3.1 ± 1.38 mV ($n = 14$) and 3.3 ± 0.98 mV ($n = 16$), respectively. The relative anion permeability of $I_{Cl(H)}$ channel was evaluated by measuring the shift in the reversal potential of $I_{Cl(H)}$ when 125 mM Cl^- in the bath solution at pH 4.65 was replaced with equimolar concentrations of other anions (Fig. 2A and B). We did not subtract the leakage current because we noticed that, in some experiments, the inward current decreased in the presence of acidic pH (see Fig. 3C), as previously reported [4]. In consequence, the absolute values of the reversal potential measured here may contain an error, but it does not change the relative permeability recorded. To determine the E_{rev}, the current to voltage values around the intercept with zero were fitted by a polynomial function. As determined from the shift in the reversal

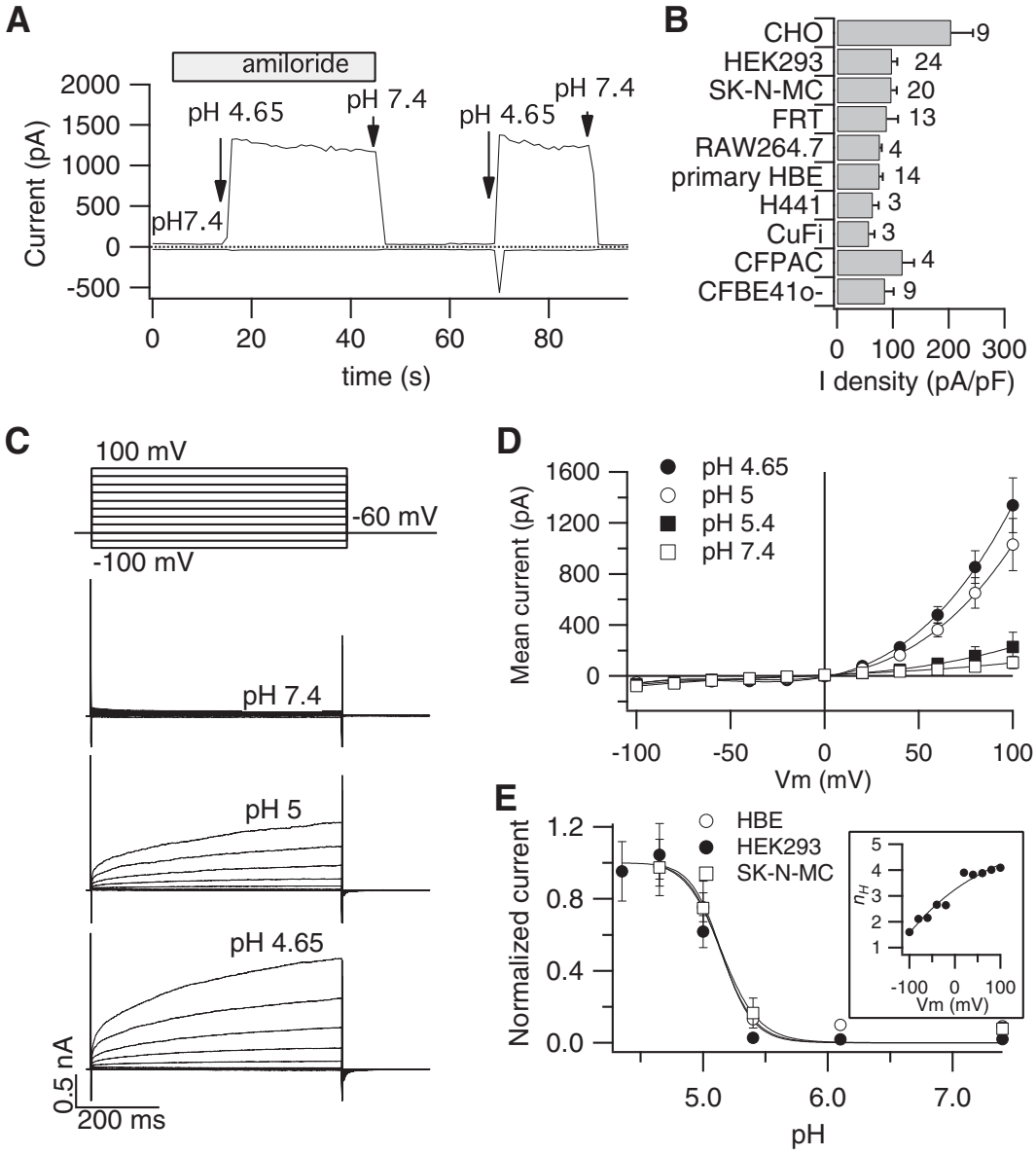

Fig. 1. The pH dependence of $I_{Cl(H)}$. (A) Time-course of current activation by acidic extracellular solution, in the presence and absence of extracellular amiloride. Continuous traces report the current recorded at −100 mV and 100 mV (lower and upper traces, respectively). (B) Density of $I_{Cl(H)}$ measured at 100 mV (pH 4.65) on different cell types. The number of experiments are indicated. (C) Stimulation protocol and representative family of currents elicited at different membrane potentials in the range between −100 to 100 mV at the indicated extracellular pH. Data are from a single HBE cell. The current to voltage relationships obtained from this experiment are shown in panel D. (E) pH dependence of currents measured at 100 mV in primary HBE, in SK-N-MC and in HEK-293 cells. The continuous lines are the best fit to a Hill equation. The inset shows the change of n_H with membrane potential.

potentials (Fig. 2B), the permeability sequence for this channel on HBE cells and in HEK-293 cells was $SCN^- > I^- > Br^- > Cl^- >$ gluconate. It is worth to note that the response to pH 4.65 of any anion was lower after iodide perfusion than before it, indicating that iodide blocks the channel. This implies a tighter binding of this anion within the pore. The voltage dependence of $I_{Cl(H)}$ channels in HBE, SK-N-MC and HEK-293 cells was determined by plotting conductance versus the applied voltage (Fig. 2C). The three curves were largely superposed. However, only HEK-293 cells could be stimulated with voltage pulses higher than 100 mV, allowing to estimate with enough confidence the voltage dependence of $I_{Cl(H)}$. On this cell line, $I_{Cl(H)}$ channels half activation point was reached at 114 ± 0.5 mV, and the charge moved was 0.51. We then analysed the tail currents generated by pulses to different voltages, following an activation pulse to 80 mV. We found that the relationship between the instantaneous current measured at the beginning of tail currents and the applied voltage is outwardly rectifying (Fig. 2D). Given that the open probability of the channel at the beginning of each test potential is the same and is equal to that at the end of the prepulse, we can conclude that the single channel conductance is intrinsically outwardly rectifying.

3.4. Pharmacology of $I_{Cl(H)}$

There are not many specific blockers for anionic channels. However, anionic channels may be characterised by their differential response to a panel of blockers. To establish the sensitivity to different compounds, $I_{Cl(H)}$ was first activated by cell perfusion with an extracellular solution at pH 4.65. Once the current was stabilised, a blocker was included in the acidic solution. Fig. 3A shows the extent of block of $I_{Cl(H)}$ channels obtained in SK-N-MC, HEK-293, HBE and CFBE41o- cells by application of 4,4'-diisothiocyano-2,2'-stilbene disulfonic acid (DIDS), niflumic acid, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB),

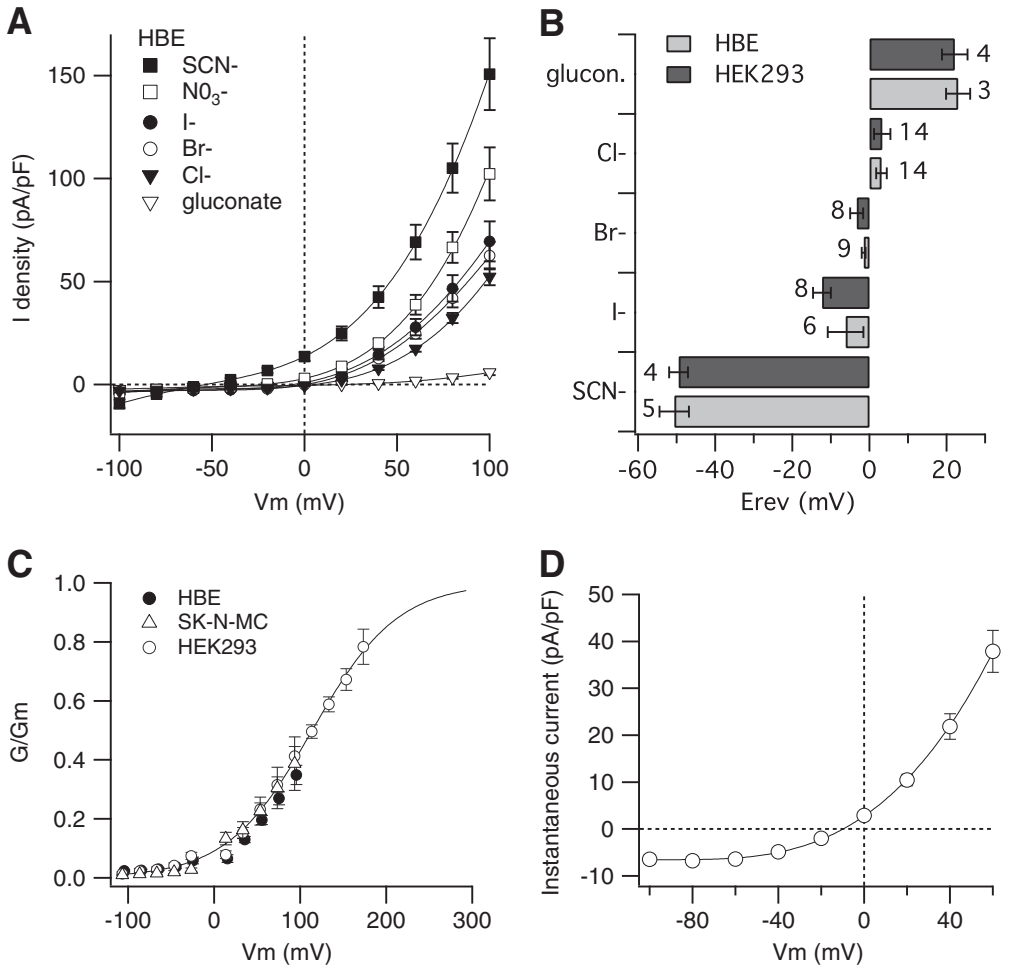

Fig. 2. Anionic permeability and voltage dependence of $I_{Cl(H)}$ channels. (A) Current density vs V_m of $I_{Cl(H)}$ in the presence of different extracellular anions measured in primary HBE cells. Data report ± SEM. The number of experiments are indicated in panel B. (B) The relative permeabilities P_X/P_{Cl} of the different anions were calculated from the shift in the reversal potential. E_{rev} was −50.6 ± 3.8 mV, −6.2 ± 4.6 mV, −0.5 ± 0.5 mV, 3.1 ± 1.4 mV and 23 ± 3.1 mV in HBE and −49.5 ± 2.43 mV, −12.3 ± 2.3 mV, −3.3 ± 1.7 mV, 3.3 ± 2.14 mV and 22.1 ± 3.3 mV in HEK-293 cells, respectively, for SCN^-, I^-, Br^-, Cl^- and gluconate. (C) Activation curve of $I_{Cl(H)}$ from HBE ($n = 14$), SK-N-MC ($n = 7$) and HEK-293 cells ($n = 14$). The continuous line is the best fit of data obtained from HEK-293 cells. (D) Outwardly rectifying instantaneous current obtained from tail currents at the beginning of pulses to different voltages, after a jump from a pulse to +80 mV. Symbols are means of 5 experiments and bars are SEM.

diphenylamine-2-carboxylate (DPC), 4,4'-dinitrostilbene-2,2'-disulfonic acid (DNDS), GlyH-101, glibenclamide and $CFTR_{Inh}$-172. The effects of these compounds resulted comparable in all cells analysed. While $I_{Cl(H)}$ was markedly inhibited (at 100 mV) by DPC, niflumic acid, NPPB and DIDS, it was only partially blocked by GlyH-101 and DNDS and was not blocked at all by glibenclamide or $CFTR_{Inh}$-172. The effects of these compounds resulted comparable in all cells analysed. The best blocker was DIDS, which reduced the current by nearly 85% at 5 μM. Even if the effect was clearer at depolarizing potentials, DIDS blocked also the inward current. A dose response with this compound showed that the apparent K_d was 0.5 μM with $n_H = 2.2$ (Fig. 3B). The block could not be reverted when DIDS was removed from the bath solution (Fig. 3C). It was necessary to return the bath pH to 7.4 for short time to fully recover the initial acid-activated current (see end of Fig. 3C).

3.5. Analysis of molecular candidates for $I_{Cl(H)}$

We investigated the possibility that $I_{Cl(H)}$ activity were due to the expression of proteins belonging to ClC or anoctamin/TMEM16 family. From the ClC family, we considered ClC-3 and ClC-7, which are expressed in HBE cells (not shown). By real-time PCR, we determined which anoctamins are expressed in the cells showing $I_{Cl(H)}$ activity. We found that HEK-293, CFPAC-1, human macrophages, and HBE cells all show expression of TMEM16F, TMEM16H, and TMEM16K (Fig. 4A). TMEM16D was also expressed in all cells except human macrophages. Even if the Ca^{2+}-dependent Cl^- channel TMEM16A was expressed in CFPAC and in HBE cells, but was not detected in HEK-293 or in macrophages, we included this protein in the analysis. In contrast, the Ca^{2+}-dependent Cl^- channel TMEM16B was undetected in macrophages, CFPAC and HBE cells and expressed at low levels in HEK-293 and, thus, was not included. We knocked down the different candidates by transfecting HEK-293 cells with the corresponding siRNAs. Then, we compared the amplitude of the response to pH 4.65 of silenced cells with those of control cells transfected with irrelevant siRNA. As shown in Fig. 4B, neither silencing the TMEM16 proteins nor ClC-3 or ClC-7 resulted in a reduction of $I_{Cl(H)}$ amplitude, even if specific mRNA levels were reduced by 78%–93%, as measured with real-time RT-PCR. As a control, we silenced TMEM16A on HBE cells and measured the channel activity in the presence of a free intracellular Ca^{2+} concentration of 325 nM. In the presence of the specific siRNA pool against TMEM16A, the current was reduced by 60%. These results indicate that the siRNAs used effectively reduced the target proteins, but none of the proteins silenced is involved in the response to extracellular acidic pH.

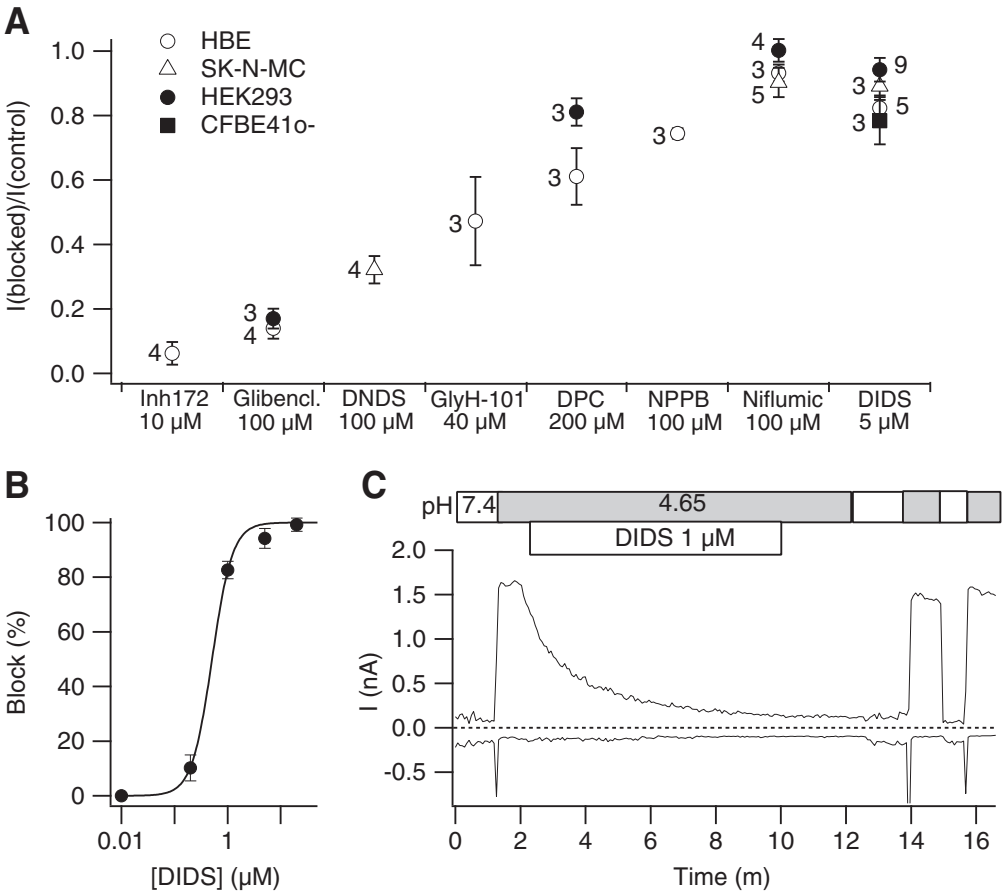

Fig. 3. Pharmacology of $I_{Cl(H)}$. (A) Blocked $I_{Cl(H)}$ fraction in the presence of the indicated compounds measured in SK-N-MC, HEK-293, primary HBE and CFBE41o- cells. The number of experiments are indicated close to respective symbols. (B) DIDS dose–response block. The continuous line is the best fit to an hyperbolic function. The apparent K_d was 0.5 μM. (C) The figure shows the time course of $I_{Cl(H)}$ measured at 100 and −100 mV as in Fig. 1. The bar above the curve indicates the changes in extracellular pH from 7.4 (white bar) to 4.65 (grey bar). Application of 1 μM DIDS to the acidic bath solution clearly blocked the outward current. However, removal of the blocker did not lead to recovery of the current. Only after a brief period at pH 7.4 the current elicited by pH 4.65 recovers the pre-block amplitude.

3.6. Intracellular mediators involved in the activation of $I_{Cl(H)}$

Although it is clear that an increase of the extracellular proton concentration leads to activation of $I_{Cl(H)}$ channel, it is not known whether this activation is the result of direct binding of protons somewhere in the channel protein or whether intracellular mediators are involved. We reasoned that if a soluble mediator is required, then the diffusion of this mediator in the large pipette volume had to cause a rundown of the current. Nevertheless, we found that the current activated by extracellular perfusion with acidic solution (pH 4.65) remained constant over more than 20 min (Fig. 5A). We can also exclude Ca^{2+} as an intracellular regulator of $I_{Cl(H)}$ since the currents could be activated at a very low Ca^{2+} concentration (~5 nM). The response to pH 4.65 was also unaffected by the presence/absence of 2 mM MgATP in the pipette solution, indicating that ATP hydrolysis is not an important step for the regulation of $I_{Cl(H)}$ channel (Fig. 5B).

The activity of many channels is regulated by intracellular mechanisms, including phosphorylation by kinases, such as PKA or PKC, calmodulin-dependent kinase, PI3K and protein tyrosine kinases (PTK). We tested the possibility that $I_{Cl(H)}$ is regulated by kinases in HEK-293 and SK-N-MC cells by using different inhibitors. These experiments were done in the presence of ATP in the intracellular solution. The concentration of inhibitors was chosen taking into account the affinities for their respective substrates and their specificities, as described in the literature. The incubation of the cells with 100 μM H7, an inhibitor of PKA and PKC [8], did not reduce the response to pH 4.65, ruling out the involvement of these kinases in the channel activation mechanism. In contrast, wortmannin and genistein, but not the inactive genistein analogue daidzein, reversible reduced $I_{Cl(H)}$ by 65 and 75%, respectively (Fig. 5C and D). These results indicate that a PI3K and a PTK are probably involved in the regulation of the channel.

We also considered the possibility that $I_{Cl(H)}$ channels were affected by exo/endocytic trafficking between the plasma membrane and intracellular vesicles. To test this hypothesis, cells were incubated at pH 7.4 for 5–10 min with dynasore, a dynamin inhibitor. Then, cells were stimulated with the acidic extracellular solution (pH 4.65) in the absence of dynasore. In this condition, the amplitude of $I_{Cl(H)}$ was ~70% lower than before dynasore application (Fig. 5C and D). After removal of dynasore, the amplitude of $I_{Cl(H)}$ slowly increased towards pre-incubation values (bottom trace in Fig. 5C). Of note, at pH 4.65 the current response to depolarizing pulses was slower immediately after dynasore removal (Fig. 5E). The time to reach half of the maximum current at 100 mV was 5.21 ± 0.44 ms and 8.84 ± 0.47 ms ($n = 7$, $p < 0.005$) for pulses before and immediately after dynasore incubation, respectively (see Fig. 5E). Probably, this difference is due to the unbinding of dynasore before the incorporation into the plasma membrane of ready-to-go vesicles containing the channels. Interestingly, also colchicine, an inhibitor of tubulin polymerization, significantly reduced $I_{Cl(H)}$ by more than 50%.

4. Discussion

Our results indicate that the properties of acid-activated Cl^- currents are quite homogenous among many different cell types including HEK-293, SK-N-MC and primary HBE cells. Similar currents, in terms of outward rectification of the current–voltage relationship and kinetics, were also observed in RAW264.7, CFPAC, CuFi, CFBE41o-, H441, FRT

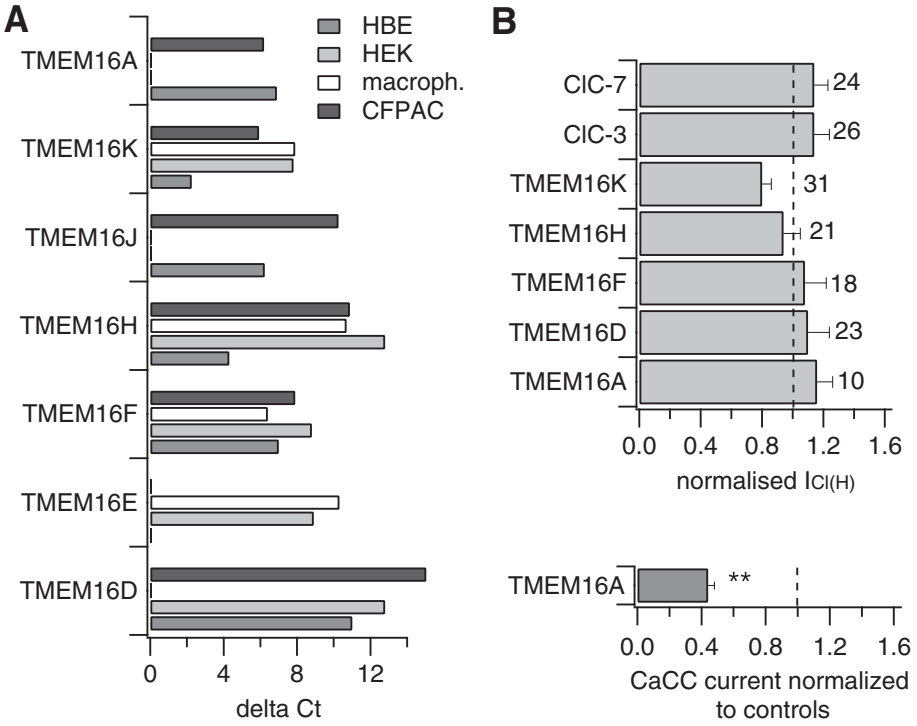

Fig. 4. Analysis of putative candidates for $I_{Cl(H)}$. (A) Analysis of expression by real-time RT-PCR of TMEM16 family members in primary HBE, HEK-293, human macrophages and CFPAC-1 cells. Data are reported as the delta Ct with respect to the reference gene (beta2-microglobulin). (B) $I_{Cl(H)}$ measured in HEK-293 cells transfected with siRNA against the indicated target and normalised to the currents measured in cells transfected with irrelevant non-targeting siRNA. The number of cells analysed is indicated. Experiments derived from at least three separate transfections. As a control, the silencing of TMEM16A in primary HBE with specific siRNA is shown. In this case, the amplitude of Ca^{2+}-activated Cl^- currents was normalised to the currents from cells transfected with non-targeting siRNA.

and CHO cells. In particular, the dependence on extracellular pH, given by an apparent half effective pH of 5.15, was almost identical in HEK-293, SK-N-MC and primary HBE cells, and in agreement with previous reports on HEK-293 cells [3,4]. Different pH sensitivities reported by others for Sertoli cells (pH 5.5,[5]), cardiac myocytes (pH 5.9, [7]), endothelial cells (pH 4.8, [8]) and HeLa cells (pH 4.5, [9]) might be ascribed to different experimental conditions, including different working temperature [17]. However, we cannot exclude that additional factors modulating the pH dependency may account for $I_{Cl(H)}$ in some of these cell lines or, alternatively, that a cell-specific subunit regulates the pH dependence of $I_{Cl(H)}$.

The relationship between $I_{Cl(H)}$ amplitude and pH indicated a Hill coefficient, n_H, near 3. This might indicate that the current activation involves the cooperative binding of a ligand, plausibly of protons. Interestingly, we found that n_H increased with V_m, suggesting either that the apparent cooperativity is higher at more depolarizing potentials, or that a multistep cascade of signalling events, leading to channel activation, accelerates with depolarization [19]. Also the activation curves of the three cell lines studied were largely superposed, supporting the idea of a single type of molecule underlying $I_{Cl(H)}$ in HEK-293, SK-N-MC and primary HBE cells.

Acid-activated Cl^- channels in HEK-293 and primary HBE cells prefer I^- and Br^- over Cl^-, indicating low field strength interaction with the channel pore. This characteristic is shared with swelling-activated anion currents [20–22] and Ca^{2+}-dependent Cl^- currents, including the recently identified TMEM16A and TMEM16B channels [23–26]. In contrast, most ClC channels are more permeable to Cl^- than to I^- [27]. Despite this critical difference, some members of the ClC family, ClC-3 and ClC-7, have been indicated as possible components of the acid-activated channel [10,11]. Therefore, we considered the possibility that ClC-3 and ClC-7 were related to acid-activated channels. As additional candidates, we also considered members of the TMEM16/anoctamin family whose role as anion channels is still undefined. Specific siRNA against ClC-3, ClC-7, TMEM16A, TMEM16D, TMEM16F, TMEM16H and TMEM16K caused a satisfying knockdown of the corresponding mRNAs. However, we found no significant change in the amplitude of $I_{Cl(H)}$, permitting us to conclude that none of these proteins is associated with the activity of the acid-activated Cl— channel. While this manuscript was under review, two groups identified LRRC8A as an essential component of the ubiquitous swelling-activated Cl^- channels [28,29]. This protein is part of a five member family and to reconstitute the typical swelling-activated current, LRRC8A requires co-transfection with other LRRC8 isoforms [29]. The knowledge about these proteins is very few thus far. However, members of this protein family are very good candidates to test as molecular correlates of $I_{Cl(H)}$.

The response to a panel of Cl^- channel blockers provided an interesting profile of pharmacological sensitivity for $I_{Cl(H)}$. Acid-activated channels were not inhibited by classical blockers of CFTR Cl^- channels such as glibenclamide and the $CFTR_{Inh}$-172. While the low-affinity and nonspecific blockers DPC, NPPB and niflumic acid inhibited $I_{Cl(H)}$ by more than 80%, the best blocker resulted DIDS, with an apparent K_d of 0.5 μM, as previously reported in HEK-293 and HeLa cells [4,9]. We have titulated DIDS finding that the p*K*a of this weak acid is 3.2. This means that at pH 7.4 and pH 4.65, 99.9% and 96.6%, respectively, are in the dissociated form. The difference is small enough to rule out the possibility that the higher affinity of DIDS for this channel than for other channels (in the 100 μM range) relies in a higher amount of the dissociated form. Curiously, withdrawal of DIDS from the acidic solution did not permit to recover the pre-blockage amplitude of $I_{Cl(H)}$. It was necessary to alkalinise the bath solution for complete reversibility of the current. The explanation might be either that it is necessary to close the pore to permit the unbinding of DIDS or that acidic pH causes a reversible modification of DIDS molecule that renders the binding irreversible. It is worth to note that the Maduke group [30] found that DIDS spontaneous hydrolysis yielded products that polymerise forming dimers, trimers, tetramers, pentamers, etc., while investigating ClC Cl^-

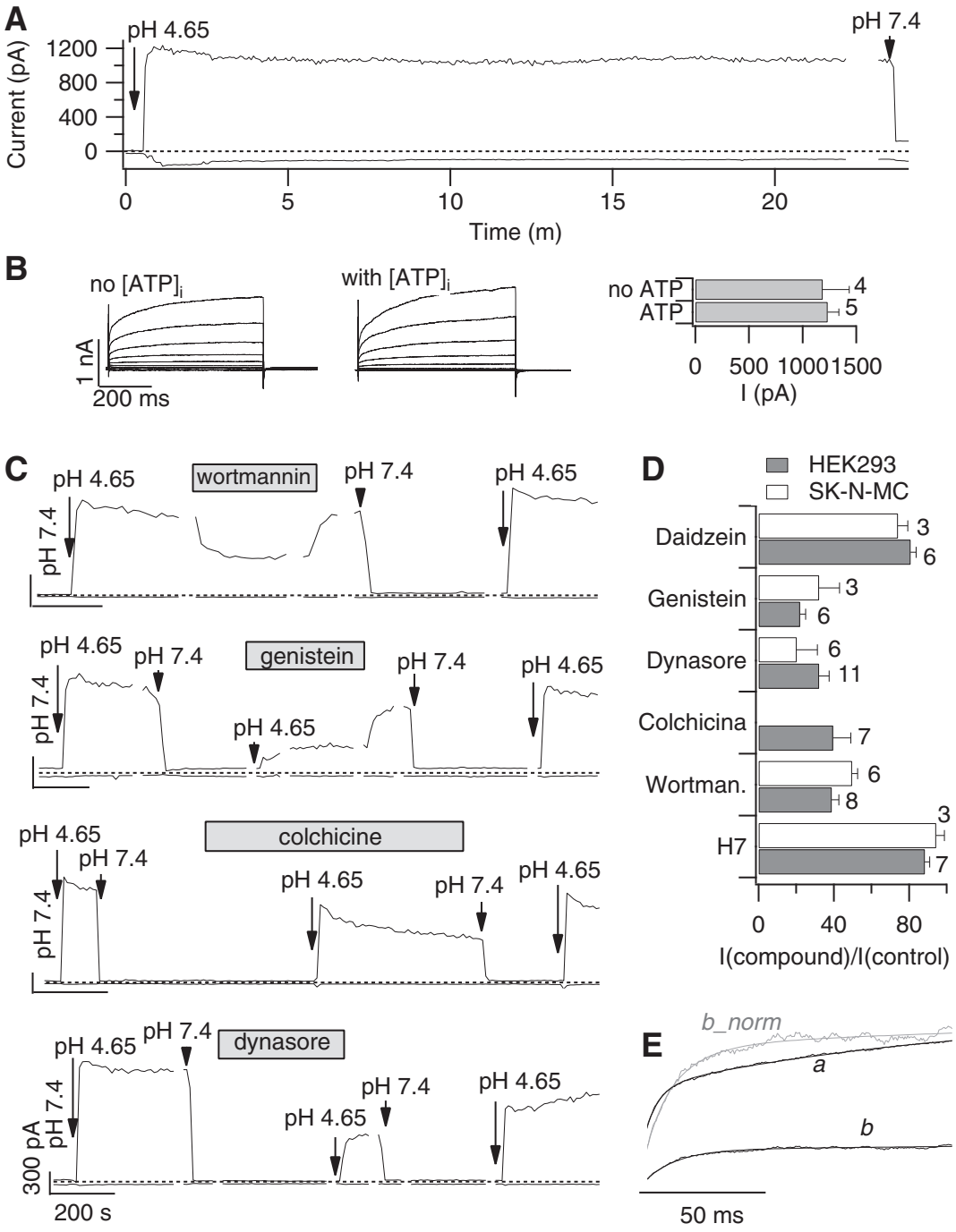

Fig. 5. Intracellular mediators involved in the regulation of $I_{Cl(H)}$. (A) The graph shows the response of HEK-293 cells to extracellular acidification measured at −100 and 100 mV. The $I_{Cl(H)}$ activated after changing extracellular pH from 7.4 to 4.65 was stable for at least 23 min, suggesting that no diffusible intracellular mediators is involved in the maintenance of $I_{Cl(H)}$. (B) Lack of $I_{Cl(H)}$ dependence on intracellular ATP (representative traces, left, and summary of currents measured at 100 mV, right). (C) Representative recordings showing the response to wortmannin (5 μM) and genistein (50 μM) applied during perfusion with the acidic bath solution. In separate experiments, dynasore (50 μM) or colchicine (100 μM) were applied in the bath solution ~10 min before reducing the pH to 4.65. Dynasore was removed before the cell stimulation with acidic pH. The amplitude of $I_{Cl(H)}$ was significantly reduced by genistein, wortmannin and dynasore, and the effect was completely reversible. The representative traces showing the effects of genistein and wortmannin were obtained in SKNMC cells while the traces showing the effect of dynasore and colchicine were from HEK-293 cells. (D) Summary of results obtained with inhibitors in SK-N-MC and HEK-293 cells. Daidzein (50 μM) and H7 (20–100 μM) were without effect. (E) Current response to a pulse to 100 mV from HEK-293 cells recorded at pH 4.65 before (*a*) and immediately after (*b*) incubation with dynasore. For comparison, (*b*) was normalized (*b_norm*) to the maximum current of trace (*a*). The first 120 ms of the traces are shown.

channel inhibitors. The affinity for ClC channels of polymeric products was higher than the affinity of DIDS and increased with polymerisation up to two orders of magnitude. In addition, the inhibition by polymers was irreversible or slowly reversible. We have not investigated for the presence of polymeric products of DIDS in our DIDS solution, but we cannot exclude that they are present.

Elucidation of the mechanisms regulating acid-activated channel function is a major challenge. Little information exists about intracellular modulators of $I_{Cl(H)}$. Lambert and Oberwinkler [4] found that the effect of acidic extracellular solution was not mediated by changes in the pH of the intracellular solution, nor by intra or extracellular calcium. Our long-lasting experiments (up to 25 min) in the nominal absence of

intracellular Ca^{2+} showed that the amplitude of $I_{Cl(H)}$ remained constant, confirming that Ca^{2+} has no role in the activation of the channel and suggesting that other diffusible intracellular mediators that might dialyse towards the pipette infinite volume are unlikely to be involved (see Fig. 5A and B). In fact, intracellular ATP did not change the current response to acidic pH, ruling out that ATP hydrolysis could regulate the channel.

We have also investigated whether activation of acid-activated channels involves amino acid phosphorylation. Many protein kinases are directly or indirectly regulated by acidic pH. For instance, autophosphorylation of MAPK increased at acidic pH (5.5–6.0). However, in this condition the catalytic activity of the kinase seems to decrease [31]. Oesophageal microvascular endothelial cells respond to acidic pH values close to those found in gastroesophageal reflux (pH 4.5) by activation of PI3K/Akt and induction of the heat shock response [32]. Autophosphorylation of PKC is favoured between pH 4 and 6, and acidic conditions can bypass the usual requirement of this kinase for calcium and lipid binding [33]. Also tyrosine kinase seems to be activated after acidification of the medium by 0.3 pH units [34]. To evaluate the possibility that some common and possibly membrane-anchored protein kinases modulate $I_{Cl(H)}$, we used general inhibitors of PKA, PKC, PI3K and PTK. The lack of effect of H7, a broad spectrum protein kinase inhibitor, ruled out the involvement of PKA and PKC-mediated phosphorylation [18]. In contrast, brief incubation of the cells with genistein, but not with the PTK inactive analogue daidzein, strongly reduced $I_{Cl(H)}$, suggesting that a PTK may be involved in the activation of the channel (see Fig. 5C and D). Also, wortmannin reversibly inhibited $I_{Cl(H)}$ indicating that a PI3K is possibly involved in channel regulation.

PI3Ks are ubiquitous enzymes that function as modulators of membrane lipids for signalling events. Interestingly, the regulatory subunit of type I PI3K, the classical signal transducing enzyme and only class of PI3Ks that can metabolize phosphatidylinositol-4,5-bisphosphate to yield phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P3), is controlled by tyrosine phosphorylated proteins [35]. PtdIns(3,4,5)P3, in turn, plays a leading role in shaping docking sites for protein effectors having the pleckstrin homology domains, including serine/threonine kinases, PTKs and GTPases such as dynamins. Although PI3Ks are usually involved in signal transduction at the plasma membrane level, some studies have also found PI3K to take part in endocytic processes and in exocytic carrier functions [36,37]. The involvement of PI3K in vesicle secretion is supported by the finding that the loss of function of p110delta catalytic isoform of PI3K either using wortmannin or RNA-mediated knockdown, abolished tumour necrosis factor exocytosis in macrophages [37].

The very acidic pH at which this channel function is never reached by the extracellular medium in physiological conditions. Only the lysosome and late endosomes, the final compartments of the endocytic pathway that contain enzymes for the degradation of proteins, lipids and polysaccharides, require a luminal pH between 4 and 6 [38,39]. Therefore, we reasoned that the acid-activated channel might be active in intracellular organelles with very acidic pH, trafficking to and from the plasma membrane where it has no functional role. Its detection there may be rather the consequence of the experimental conditions. In this case, the involvement of PI3K might be an evidence of the traffic of the channel in exo/endocytosis.

To further define whether wortmannin effect on acid-activated channel was mediated by the disruption of trafficking events, and considering that the channel response to acidic pH could be regulated by dynamin-mediated endocytosis of regions of the plasma membrane, we turned to dynasore, a cell-permeable inhibitor of dynamins. Our hypothesis was that disruption of dynamin activity by dynasore would lead to a reduction of vesicle endocytosis and therefore an increase of $I_{Cl(H)}$. Surprisingly, the effect was opposite. Brief incubation with dynasore reversibly reduced the amplitude of $I_{Cl(H)}$ by 70% (Fig. 5C and D). Dynamins are proteins best known as controllers of fission in receptor-mediated endocytosis, synaptic vesicle recycling, caveolae internalization, and vesicle trafficking in and out of the Golgi [40–42]. However, recent investigations have found that dynamins are also involved in the regulation of immediate post-fusion events in exocytosis [43]. Using a dynamin mutant with reduced GTPase activity or incubating the cells with dynasore, these authors found that expansion of the fusion pore was slowed down. In contrast, when cells were transfected with a dynamin mutant with elevated GTPase activity, fusion pore expansion was accelerated indicating that dynamin could directly alter the dynamics of exocytosis. Therefore, our result using dynasore probably indicate that the acid-activated channel arrives to the plasma membrane trafficking through an exocytic process and that in our cells the role of dynamin in the fusion pore expansion of $I_{Cl(H)}$-containing vesicles is higher than its role in endocytosis. Alternatively, it is possible to speculate that the very low pH is the stimulus that induces fusion of $I_{Cl(H)}$-containing vesicles perhaps after phosphorylation of PTK, PI3K and activation of dynamins. Something similar has been found before in ventricular cardiomyocytes, where K_{ATP} channels were found to be retained in the Golgi until the arrival of beta-adrenergic stimulation. This stimulus leads to the activation of PKA and incorporation of the channels into the plasma membrane [44]. The hypothesis that $I_{Cl(H)}$ channels are contained in vesicles attached to the plasma membrane is supported by the finding that colchicine reduced the current amplitude at pH 4.65. This result also indicates that the vesicles are probably anchored to the membrane by a mechanism involving microtubules.

5. Conclusions

The data presented here provide evidence that using the same experimental conditions, $I_{Cl(H)}$ from three distinct cell lines show identical characteristics, including pH-dependence, anionic permeability and voltage-dependence, sensitivity to blockers and to modulators. This strongly points to this current being the manifestation of a unique protein or protein complex. Silencing experiments demonstrated that neither ClC-3, nor ClC-7, TMEM16A, TMEM16D, TMEM16F, TMEM16H or TMEM16K are related to $I_{Cl(H)}$. While the mechanism of activation of $I_{Cl(H)}$ is still unclear, Ca^{2+}, ATP, and phosphorylation by PKA or PKC do not seem to be implicated. On the other hand, we have shown here that a PI3K and a PTK are indeed necessary for complete channel activation. In addition, insertion of vesicle membranes containing the channel seems a mechanism involved in the activation/recycling of $I_{Cl(H)}$, as suggested by the inhibition by dynasore. While it is clear that more experiments are needed, this study provides 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.

Acknowledgements

This work was supported by Ministero della Salute (Ricerca Finalizzata: GR-2009-1596824; Ricerca Corrente: Cinque per mille).

References

[1] C. Duran, C.H. Thompson, Q. Xiao, H.C. Hartzell, Chloride channels: often enigmatic, rarely predictable, Annu. Rev. Physiol. 72 (2010) 95–121.
[2] T. Jentsch, T. Maritzen, A.A. Zdebik, Chloride channel diseases resulting from impaired transepithelial transport or vesicular function, J. Clin. Invest. 115 (2005) 2039–2046.
[3] M. Nobles, C.F. Higgins, A. Sardini, Extracellular acidification elicits a chloride current that shares characteristics with ICl(swell), Am. J. Physiol. Cell Physiol. 287 (2004) C1426–C1435.
[4] S. Lambert, J. Oberwinkler, Characterization of a proton-activated, outwardly rectifying anion channel, J. Physiol. 567 (2005) 191–213.
[5] C. Auzanneau, V. Thoreau, A. Kitzis, F. Becq, A Novel voltage-dependent chloride current activated by extracellular acidic pH in cultured rat Sertoli cells, J. Biol. Chem. 278 (2003) 19230–19236.
[6] C.Y. Shi, R. Wang, C.X. Liu, H. Jiang, Z.Y. Ma, L. Li, W. Zhang, Simvastatin inhibits acidic extracellular pH-activated, outward rectifying chloride currents in RAW264.7

monocytic-macrophage and human peripheral monocytes, Int. Immunopharmacol. 9 (2009) 247–252.
[7] S. Yamamoto, T. Ehara, Acidic extracellular pH-activated outwardly rectifying chloride current in mammalian cardiac myocytes, Am. J. Physiol. Heart Circ. Physiol. 290 (2006) H1905–H1914.
[8] Z.Y. Ma, W. Zhang, L. Chen, R. Wang, X.H. Kan, G.Z. Sun, C.X. Liu, L. Li, Y. Zhang, A proton-activated, outwardly rectifying chloride channel in human umbilical vein endothelial cells, Biochem. Biophys. Res. Commun. 371 (2008) 437–440.
[9] H.Y. Wang, T. Shimizu, T. Numata, Y. Okada, Role of acid-sensitive outwardly rectifying anion channels in acidosis-induced cell death in human epithelial cells, Pflugers Arch. 454 (2006) 223–233.
[10] J.J. Matsuda, M.S. Filali, M.M. Collins, K.A. Volk, F.S. Lamb, The ClC-3 Cl−/H + antiporter becomes uncoupled at low extracellular pH, J. Biol. Chem. 285 (2010) 2569–2579.
[11] K. Ohgi, F. Okamoto, H. Kajiya, R. Sakagami, K. Okabe, Antibodies against ClC7 inhibit extracellular acidification-induced Cl^- currents and bone resorption activity in mouse osteoclasts, Naunyn Schmiedeberg's Arch. Pharmacol. 383 (2011) 79–90.
[12] Z.J. Fu, X.Z. Li, Q.R. Wang, L. Shi, L.Q. Zhang, X.L. Pan, Extracellular acidic pH-activated, outward rectifying chloride currents can be regulated by reactive oxygen species in human THP-1 monocytes, Biochem. Biophys. Res. Commun. 432 (2013) 701–706.
[13] M. Kraus, B. Wolf, Implications of acidic tumour microenvironment for neoplastic growth and cancer treatment: a computer analysis, Tumour Biol. 17 (1996) 133–154.
[14] Z.G. Xiong, G. Pignataro, M. Li, S.Y. Chang, R.P. Simon, Acid-sensing ion channels (ASICs) as pharmacological targets for neurodegenerative diseases, Curr. Opin. Pharmacol. 8 (2008) 25–32.
[15] W.F. Fleet, T.A. Johnson, C.A. Graebner, L.S. Gettes, Effect of serial brief ischemic episodes on extracellular K^+, pH, and activation in the pig, Circulation 2 (1985) 922–932.
[16] A. Lardner, The effects of extracellular pH on immune function, J. Leukocyte Biol. 69 (2001) 522–530.
[17] K. Sato-Numata, T. Numata, T. Okada, Y. Okada, Acid-sensitive outwardly rectifying (ASOR) anion channels in human epithelial cells are highly sensitive to temperature and independent of ClC-3, Pflugers Arch. 465 (2013) 1535–1543.
[18] H. Hidaka, M. Inagaki, S. Kawamoto, Y. Sasaki, Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C, Biochemistry 23 (1984) 5036–5041.
[19] A. Sorribas, B. Hernández-Bermejo, E. Vilaprinyo, R. Alves, Cooperativity and saturation in biochemical networks: a saturable formalism using Taylor series approximations, Biotechnol. Bioeng. 97 (2007) 1259–1277.
[20] P.S. Jackson, K. Strange, Characterization of the voltage-dependent properties of a volume-sensitive anion conductance, J. Gen. Physiol. 105 (1995) 661–676.
[21] T. Voets, G. Droogmans, B. Nilius, Modulation of voltage-dependent properties of a swelling-activated Cl− current, J. Gen. Physiol. 110 (1997) 313–325.
[22] O. Zegarra-Moran, L.J. Galietta, Biophysical characteristics of swelling-activated Cl − channels in human tracheal 9HTEo-cells, J. Membr. Biol. 165 (1998) 255–264.
[23] M.G. Evans, A. Marty, Calcium-dependent chloride currents in isolated cells from rat lacrimal glands, J. Physiol. 378 (1986) 437–460.
[24] Z. Qu, H.C. Hartzell, Anion permeation in Ca2+-activated Cl− channels, J. Gen. Physiol. 116 (2000) 825–844.
[25] B.C. Schroeder, T. Cheng, Y.N. Jan, L.Y. Jan, Expression cloning of TMEM16A as a calcium-activated chloride channel subunit, Cell 134 (2008) 1019–1029.
[26] S. Pifferi, M. Dibattista, A. Menini, TMEM16B induces chloride currents activated by calcium in mammalian cells, Pflugers Arch. 458 (2009) 1023–1038.
[27] T.J. Jentsch, V. Stein, F. Weinreich, A.A. Zdebik, Molecular structure and physiological function of chloride channels, Physiol. Rev. 82 (2002) 503–568.
[28] Z. Qiu, A.E. Dubin, J. Mathur, B. Tu, K. Reddy, L.J. Miraglia, J. Reinhardt, A.P. Orth, A. Patapoutian, SWELL1, a plasma membrane protein, is an essential component of volume-regulated anion channel, Cell 157 (2014) 447–458.
[29] F.K. Voss, F. Ullrich, J. Münch, K. Lazarow, D. Lutter, N. Mah, M.A. Andrade-Navarro, J.P. von Kries, T. Stauber, T.J. Jentsch, Identification of LRRC8 heteromers as an essential component of the volume-regulated anion channel VRAC, Science 344 (2014) 634–638.
[30] K. Matulef, A.E. Howery, L. Tan, W.R. Kobertz, J. Du Bois, M. Maduke, Discovery of potent CLC chloride channel inhibitors, ACS Chem. Biol. 3 (2008) 419–428.
[31] A.A. Tokmakov, K.-I. Sato, Y. Fukami, Deregulation of mitogen-activated protein kinase at low pH due to a structural rearrangement of activation segment, Biochim. Biophys. Acta 1476 (2000) 66–74.
[32] P. Rafiee, M.E. Theriot, V.M. Nelson, J. Heidemann, Y. Kanaa, S.A. Horowitz, A. Rogaczewski, C.P. Johnson, I. Ali, R. Shaker, D.G. Binion, Human esophageal microvascular endothelial cells respond to acidic pH stress by PI3K/AKT and p38 MAPK-regulated induction of Hsp70 and Hsp27, Am. J. Physiol. Cell Physiol. 291 (2006) C931–C945.
[33] P.N. McFadden, A. Mandpe, D. Koshland Jr., Calcium-and lipid-independent protein kinase C autophosphorylation Activation by low pH, J. Biol. Chem. 264 (1989) 12765–12771.
[34] Y. Yamaji, O.W. Moe, R.T. Miller, R.J. Alpern, Acid activation of immediate early genes in renal epithelial cells, J. Clin. Invest. 94 (1994) 1297–1303.
[35] M. Bohdanowicz, S. Grinstein, Role of phospholipids in endocytosis, phagocytosis, and macropinocytosis, Physiol. Rev. 93 (2013) 69–106.
[36] S.M. Jones, K.E. Howell, Phosphatidylinositol 3–Kinase is required for the formation of constitutive transport vesicles from the TGN, J. Cell Biol. 139 (1997) 339–349.
[37] P.C. Low, R. Misaki, K. Schroder, A.C. Stanley, M.J. Sweet, R.D. Teasdale, B. Vanhaesebroeck, F.A. Meunier, T. Taguchi, J.L. Stow, Phosphoinositide 3-kinase δ regulates membrane fission of Golgi carriers for selective cytokine secretion, J. Cell Biol. 190 (2010) 1053–1065.
[38] C.S. Pillay, E. Elliott, C. Dennison, Endolysosomal proteolysis and its regulation, Biochem. J. 363 (2002) 417–429.
[39] F.R. Maxfield, D.J. Yamashiro, Endosome acidification and the pathways of receptor-mediated endocytosis, Adv. Exp. Med. Biol. 225 (1987) 189–198.
[40] J.E. Hinshaw, Dynamin and its role in membrane fission, Annu. Rev. Cell Dev. Biol. 16 (2000) 483–519.
[41] A.-C. Durieux, B. Prudhon, P. Guicheney, M. Bitoun, Dynamin 2 and human diseases, J. Mol. Med. 88 (2010) 339–350.
[42] S.-A. Chan, B. Doreian, C. Smith, Dynamin and myosin regulate differential exocytosis from mouse adrenal chromaffin cells, Cell. Mol. Neurobiol. 30 (2010) 1351–1357.
[43] A. Anantharam, M.A. Bittner, R.L. Aikman, E.L. Stuenkel, S.L. Schmid, D. Axelrod, R.W. Holz, A new role for the dynamin GTPase in the regulation of fusion pore expansion, Mol. Biol. Cell 22 (2011) 1907–1918.
[44] E.C. Arakel, S. Brandenburg, K. Uchida, H. Zhang, Y.W. Lin, T. Kohl, B. Schrul, M.S. Sulkin, I.R. Efimov, C.G. Nichols, S.E. Lehnart, B. Schwappach, Tuning the electrical properties of the heart by differential trafficking of K_{ATP} ion channel complexes, J. Cell Sci. 127 (2014) 2106–2119.