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Review

Cell volume regulation and swelling-activated chloride channels

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Abstract

Maintenance of a constant volume is essential for normal cell function. Following cell swelling, as a consequence of reduction of extracellular osmolarity or increase of intracellular content of osmolytes, animal cells are able to restore their original volume by activation of potassium and chloride conductances. The loss of these ions, followed passively by water, is responsible for the homeostatic response called regulatory volume decrease (RVD). Activation of a chloride conductance upon cell swelling is a key step in RVD. Several proteins have been proposed as candidates for this chloride conductance. The status of the field is reviewed, with particular emphasis on ClC-3, a member of the ClC family which has been recently proposed as the chloride channel involved in cell volume regulation.

Keywords: Regulatory volume decrease (RVD); Cell swelling-activated chloride current ($I_{Cl,swell}$); Chloride channel ClC

1. Introduction

Regulation of cell volume is an essential function that cells have conserved throughout evolution in order to maintain constant cellular volume and to regulate internal composition in response to changes of osmolarity of the extracellular medium [1]. The majority of mammalian cells have retained this function (for recent general reviews on cell volume regulation see Refs. [2,3]). Although most mammalian cells live in a very tightly regulated and controlled environment, they can face both external and internal osmotic challenges. For example, intestinal epithelial cells, during absorption, are exposed to anisosmotic luminal fluids and cells in the kidney medulla have to experience prolonged periods of very high extracellular osmolarity as well as rapid change of osmolarity [2]. Changes in intracellular concentrations of osmolytes, as consequence of substrate transport or alterations of metabolic rates, also constitute a challenge to the constancy of cell volume. For example, hepatocytes change cell volume in response to the uptake of large quantities of amino acids and other nutrients [4], and epithelial cells, involved in transepithelial transport, have to balance substrate intake [5]. Furthermore, osmolyte concentration can also be affected by changes of metabolic rates such as glycogen breakdown in hepatocytes [6].

Why is the constancy of cell volume important? Metabolic rates are influenced by the concentrations of enzymes and co-factors in the cell; rates of substrate transport are strictly dependent on the maintenance of electrical driving force that is influenced by the intracellular ion concentrations; receptor recycling, hormone and transmitter release, excitability and contraction, migration, pathogen–host interactions, cell proliferation and cell death are also influenced by changes in cell volume. As well as this plethora of normal functions, pathological situations such as brain injuries and apoptosis are also dependent on cell volume. For example, following ischaemia or trauma, loss of cell volume control and consequent nerve cell swelling in a rigid structure such as the skull, demonstrates that tight regulation of cell volume is of paramount importance not just for optimal functioning but for survival [7,8]. Apoptotic cell volume decrease has also been associated with regulatory volume decrease (RVD) since the decrease of cell volume

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after apoptotic stimuli is due to activation of the same potassium and chloride channels that are responsible for physiological RVD [9–11].

How is cell volume regulated in the face of osmotic challenges [2,12]? In isosmotic conditions, mammalian cells have a tendency to swell because they contain impermeant anionic macromolecules, mostly proteins and organic phosphates, which create an osmotic pressure: the Donnan effect. The cells counteract this colloid-osmotic swelling by a net osmolyte efflux through the activity of the Na^+/K^+ pump [12]. In the presence of anisosmotic conditions, a consequence of rapid alteration of the intra or extracellular osmolality, the passive flow of water causes cell swelling or shrinkage. The cell restores its original volume by activating channels or transporters in order to lose or gain osmolytes as appropriate, typically K^+, Cl^- and organic osmolytes. This induces a directional water flow and restores the cell volume. The decrease of cell volume after cell swelling is referred to as RVD, and the increase of cell volume after cell shrinkage as regulatory volume increase (RVI). Although transport pathways other than K^+ and Cl^- channels have been implicated in the phenomenon of RVD, as for example the K^+–Cl^- co-transport system [13], shown to be sufficient to mediate RVD in rat thymocytes [14], in most cell types tested, the coordinated action of K^+ and Cl^- channels is the principal mechanism for RVD. As it has been shown recently for an human bronchial epithelial cell line, we may envisage that cell swelling activates, a calcium conductance [15] and the consequent rise in intracellular calcium is responsible for the activation of calcium-activated K^+ channels [16]. In addition to calcium-activated K^+ channels, cell swelling has, in other cell types, been reported to be associated either with voltage-dependent K^+ channels of different types [17] or with two pore-domain K^+ channels [18]. In order to maintain electroneutrality, the efflux of K^+ is paralleled by the efflux of Cl^- mediated by a swelling-activated chloride channel.

This review focuses on the functional and molecular nature of this elusive chloride channel.

2. How to measure cell volume changes

Several techniques are available to estimate cell volume changes in cell populations and in single cells. Detection of

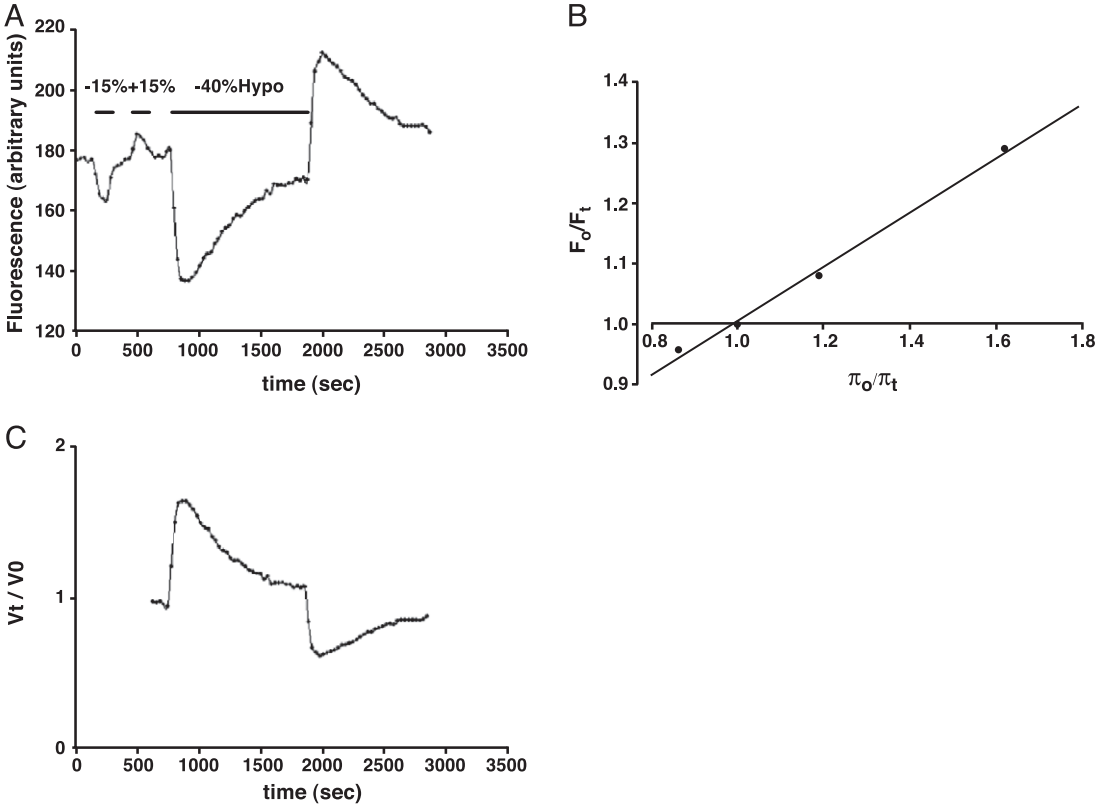

Fig. 1. Measurement of volume change in a single HEK293 cell. Cells were imaged by confocal microscopy as described previously [91]. Panel A: average fluorescence signal of a cellular area plotted against the time from a confocal optical section. Data points acquired every 30 s. To calibrate the fluorescence signal, brief exposures to 15% hypotonic and 15% hypertonic solutions were used. Upon exposure of the cell to 40% hypotonic solution, the fluorescent signal decreased suddenly and recovered its original intensity following a different kinetic. Panel B: relationship between the reciprocal of the steady-state changes in fluorescence (F_0/F_t) and the reciprocal of the external osmotic pressure (π_0/π_t) for the cell shown in panel A. The slope of the linear fitting is 0.447 (correlation coefficient 0.997) giving a background fluorescence $F_{bkg}=0.55$. Panel C: the fluorescent signal shown in panel A was analyzed and converted to relative volume measurements [26]. RVD is evident upon exposure to 40% hypotonic solution.

changes in cell volume for a cell population with a good time resolution can be followed using a Coulter counter, based on electronic cell sizing [19], or by flow cytometry that estimates cell diameter by the forward-angle of light scatter [20]. Their use is widespread and particularly useful for cells in suspension. Other methods have been developed for measuring cell volume changes of adherent cells. Estimation of the cell diameter simply by detection of its edges in bright field images, or by more sophisticated optical techniques such as phase contrast or Nomarski microscopy, have been successfully employed to measure the changes of cell volume after osmotic challenges [21,22]. In the case of confluent layers of cylindrical epithelial cells, cell thickness has been used as an index of cell volume. Measuring the change of their major cellular axis was achieved by covering the apical membrane with fluorescent biotin-loaded microbeads and following their position after hypotonic challenge [23]. More recently, scanning acoustic microscopy (SAM) [24] has been used to follow volume regulation after hypotonic shock by detecting changes in mechanical properties of the cells [25].

Alvarez-Leefmans et al. [26] have established a technique to estimate changes in cell volume as a function of changes in concentration, and therefore signal intensity of a cell-impermeant fluorescence dye. Briefly, cells are exposed to calcein-AM (Molecular Probes), a non-fluorescent and membrane-permeant dye. Calcein-AM, after having entered the cells, is converted to calcein by removal of the acetoxymethyl group (AM) by intracellular esterases. Calcein is fluorescent and cell-impermeant, and therefore remains trapped inside the cell. When cells are exposed to hypotonic solution they swell, increase their volume, thus decreasing the concentration of calcein and, consequently, the intensity of its fluorescent signal in a manner directly proportional to the dye concentration. The opposite happens when they are exposed to a hypertonic solution. The fluorescence signal is calibrated by brief exposures of the calcein-loaded cells to, for example, 15% hypotonic and 15% hypertonic solutions and the average signal intensity from a small volume within the cell is detected by confocal microscopy (Fig. 1, panel A). Fig. 1, panel B shows that the relationship between the reciprocal of the relative steady-state changes in fluorescence (F_0/F_t) for a HEK293 cell and the reciprocal of the relative osmotic pressure of the extracellular medium (π_o/π_t) is linear within the range of osmolarity tested. The linear relationship (correlation coefficient 0.997) confirms that changes in cell volume are reflected in changes in calcein concentration. The linear slope in this case was 0.447, differing from the unitary slope expected for an ideal osmometric behaviour. This discrepancy is presumed due to calcein bound to cell structures, referred to as background fluorescence, and thus unresponsive to change in cell volume. The relative changes in volume (V_t/V_o) after challenge with anisosmotic solutions could therefore be computed from F_0/F_t following the derivation proposed by Alvarez-Leefmans et al. [26], which takes into account the background fluorescence. Fig. 1, panel C shows graphically the result of this operation.

3. The phenomenon of RVD

Fig. 1, panel C shows that the volume of a HEK293 cell increases rapidly after challenge with a hypotonic solution (40% reduction of the osmolarity), reaches a maximum value and, with a different kinetic, recovers its original volume still in the presence of the hypotonic solution: this is the phenomenon of RVD. If the cell is abruptly returned to isotonic solution, we observe a sudden decrease of volume followed by a RVI post-RVD [12,27].

RVD is the result of the concerted activity of K^+ and Cl^- channels [12,27]. The loss of osmolytes, following hypotonic challenge, induces a passive efflux of water with the consequent decrease of cell volume. This regulatory action is brought about by the cell in order to maintain a constant volume in the face of changes in osmolarity, for example by an experimental decrease of the osmolarity of the extracellular medium. This homeostatic reaction maintains a constant intracellular environment that is strictly regulated to optimise vital functions. The decrease of cell volume and the phenomenon of RVI observed after the cell has gone through RVD, can be explained simply by considering that the cell during RVD has lost osmolytes, and when returned to an isosmotic solution, the cell experiences it as hypertonic, thus water leaves the cell with consequent volume decrease. Here again, the cell activates a homeostatic response, RVI, to bring the cell volume to its original value.

4. On the molecular identity of the swelling-activated chloride channels involved in RVD

In order to exhibit RVD, the cell has to decrease its content of osmolytes so that an efflux of water is induced. Although activation of KCl co-transporters is sometimes a consequence of hypotonic challenge [13], activation of K^+ and Cl^- channels has been detected in a large majority of cells tested. The coordinated action of K^+ and Cl^- channels brings about the efflux of KCl and the consequent water efflux due to intracellular reduction of osmolytes.

Activation of a Cl^- leak pathway by cell swelling was first demonstrated in Ehrlich ascites tumour cells [28], and in lymphocytes [29], and subsequently found in essentially all cell types investigated. The biophysical characteristics of the Cl^- current activated upon hypotonic challenge, $I_{Cl,swell}$, are well-described and remarkably constant in different cell types [30–32]. Fig. 2, panel A shows a typical recording of $I_{Cl,swell}$ in a HEK293 cell. The current reaches a steady-state level of activation after 4–6 min in hypotonic solution, presents time-dependent inactivation at depolarised potentials and is moderately outwardly rectifying (Fig. 2, panel B). The degree and rate of the depolarization-induced

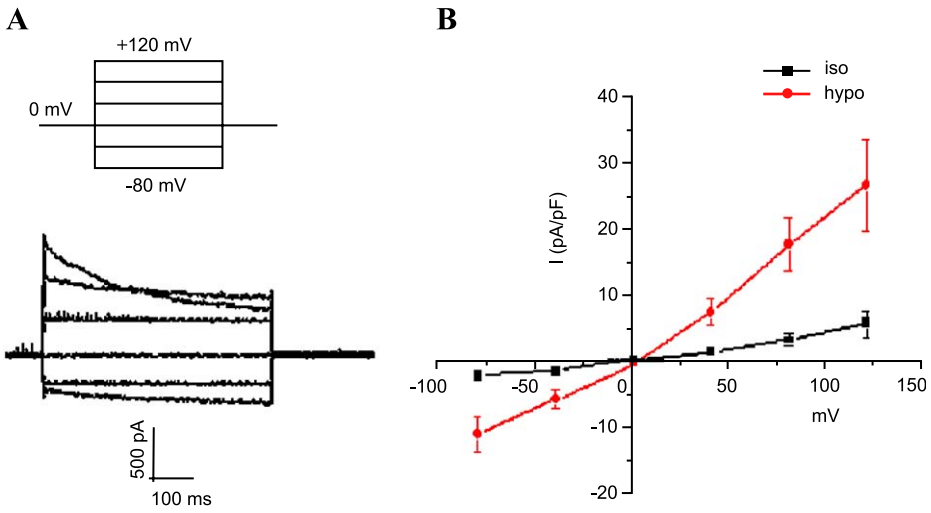

Fig. 2. Recording of $I_{Cl,swell}$. Chloride currents were measured in the whole cell recording mode of the patch clamp technique as described previously [47,50]. Panel A: a family of chloride currents after exposure to 30% hyposmotic solution is shown (bottom). HEK293 cell was stimulated with square voltage pulses from − 80 to + 120 mV in 40-mV steps from a 0 mV holding potential (as shown at the top of the panel). Panel B: I/V relationships in the presence of isotonic and hypotonic solutions. The elicited peak currents are computed against the pulse voltage. The currents recorded in hypotonic solution showed the typical outwardly rectifying behaviour.

inactivation is rather variable, being, e.g. extensive in rat C6 glioma cells [33], moderate in Ehrlich cells [34], and essentially absent in Jurkat lymphocytes [35]. $I_{Cl,swell}$ has a typical selectivity: $I^->Br^->Cl^->F^->$ gluconate that corresponds to Eisenman's sequence I. It is blocked by NPPB, DIDS, A9C, niflumic acid, the oestrogen receptor antagonist tamoxifen and by DCPIB, an indanone compound that has been shown to be the most selective inhibitor so far [36]. Its activity is dependent on cytosolic ATP, but not its hydrolysis, suggesting a non-hydrolytic binding of ATP to the channel. However, its ATP dependence varies with the rate of cell swelling [37]. On the contrary, extracellular ATP blocks $I_{Cl,swell}$ in a well-known voltage-dependent manner.

Despite this extensive biophysical characterization of $I_{Cl,swell}$, the molecular identity of the channel is still a matter of debate (for a recent review see Ref. [36]). Several candidates have been suggested and tested.

4.1. P-glycoprotein

P-glycoprotein (P-gp), a member of the ATP binding cassette (ABC) superfamily of transporters, is an ATP-dependent transporter of amphipathic substrates. Its overexpression in tumour cells is one of the major causes of the phenomenon of multidrug resistance (MDR). P-gp transports chemotherapeutic drugs out of the cell, reducing their intracellular concentration below their cytotoxic threshold and therefore inducing resistance. P-gp is also a regulator of $I_{Cl,swell}$. This is not entirely surprising because, although P-gp is clearly a transporter, other members of the ABC superfamily such as cystic fibrosis transmembrane conductance regulator (CFTR) and sulfonylurea receptor (SUR) are channels and/or regulators of channels. CFTR has been shown to be a chloride channel itself [38] and a regulator of different conductances [39], while SUR is a regulator of Kir6.2, an ATP-sensitive K^+ channel [40]. P-gp was proposed as a candidate for the $I_{Cl,swell}$, or a component thereof, since an association between P-gp expression and cell-swelling activated chloride channels was shown [41,42]. This hypothesis was initially the subject of some controversy, and it is clear now that P-gp is not the channel responsible for $I_{Cl,swell}$ but instead regulates the activity of an endogenous swelling-activated chloride channel. The initial controversy arose for several reasons. Firstly several inhibitors of $I_{Cl,swell}$ are also inhibitors of P-gp activity [41–45] although it has been shown also that many of these compounds block the $I_{Cl,swell}$ even in cells not expressing P-gp [43,46]. The comparability of the pharmacological profiles suggests either that a similar binding site is present in P-gp and $I_{Cl,swell}$ channels or that P-gp and $I_{Cl,swell}$ have a protein in common which binds these compounds and inhibits both P-gp and $I_{Cl,swell}$. Secondly P-gp expression does not influence the magnitude of $I_{Cl,swell}$ but instead increases the sensitivity of the channel to hyposmotic solution [47–49] and its rate of activation [47,48,50]. This means that in cells expressing P-gp, a small decrease of extracellular osmolarity (10%) will more promptly elicit a swelling-activated current than in non-P-gp expressing cells. However, in response to a larger osmotic gradient, no difference in the magnitude of the swelling-activated current is detectable between P-gp expressing and non-expressing cells [51,52]. The rate of activation of $I_{Cl,swell}$ upon hypotonic challenge is slow and takes several minutes to reach the steady-state. This length of time is shortened if the cell expresses P-gp. However, the maximum current that can be elicited is not influenced by P-gp presence when the steady-state has been reached. This is a possible explanation for the failure of other studies to detect an effect of P-gp on channel

activation [53–55]. In conclusion, P-gp is not a chloride channel by itself: $I_{Cl,swell}$ can be measured in several cell types that do not express significant level of P-gp [46,54,56–58]. Furthermore, the endogenous swelling-activated chloride current that disappears several days after defolliculation in *Xenopus* oocytes cannot be restored by expression of P-gp [59]. P-gp instead, as discussed above, affects the rate and the sensitivity of $I_{Cl,swell}$.

4.2. pI_{Cln}

The association of pICln with $I_{Cl,swell}$ has also been controversial [31,60]. pICln was identified by expression cloning in *Xenopus laevis* oocytes. Since its expression was shown to induce an outwardly rectifying chloride current, blocked by extracellular nucleotides and elicited in the absence of any osmotic challenges, pICln was proposed as a chloride channel [61]. Subsequently, pICln was shown to be localized mainly in the cytoplasm [62]. Translocation and insertion of pICln into the plasma membrane upon exposure to hypotonicity was proposed as the mechanism of activation [63]. Successive investigations failed to confirm this finding [64,65] although recently, by employing FRET measurements, translocation to the plasma membrane of pICln, following exposure to hypotonic solution, has been detected in NIH/3T3 fibroblasts [66]. Furthermore, it was shown that the chloride current induced by expression of pICln, in *Xenopus* oocytes, differed from the endogenous $I_{Cl,swell}$ and that expression of either pICln or the unrelated protein ClC-6 generated identical chloride conductance in *Xenopus* oocytes [65,67]. In addition, the extracellular binding site of pICln responsible for nucleotide inhibition was not confirmed [68]. pICln has also been purified and reconstituted into artificial lipid bilayers generating a cation selective channel [69] whose relative permeability of cations over anions depends on the presence of calcium [70]. However, its reconstitution in heart-lipid extract generated a chloride channel [71]. pICln seems therefore able to generate currents by itself. It remain to be proven whether it really has channel activity in vivo and what its relationship, if any, is to $I_{Cl,swell}$.

4.3. ClC channels: ClC-2 and ClC-3

The ClC family was initially identified by expression cloning of a voltage-gated chloride channel from the electric organ of the ray *Torpedo marmorata* [72]. ClC channels are present from prokaryote to eukaryote organisms. There are nine known ClC homologues in mammals designated successively from ClC-1 to ClC-7, plus two kidney-specific ClC channels, ClC-Ka and ClC-Kb [73–75]. Among these channels, ClC-2 and ClC-3 have been reported to be activated by cell swelling.

ClC-2, cloned by homology with ClC-1, is activated by cell swelling [76], hyperpolarization [77] as well as by extracellular acidic solutions [78]. Its N-terminal portion has been identified as essential for the mechanism of volume-sensitive gating [76]. Therefore, ClC-2 is a chloride channel where the sensor for cell swelling is a portion of the channel itself. Its biophysical properties, namely its inwardly rectifying I/V relationship and anion selectivity as well as its lack of inhibition by DIDS, differ from the previously mentioned characteristics of $I_{Cl,swell}$. Although these exclude ClC-2 as the channel responsible for the swelling-activated chloride current, ClC-2 has nevertheless been implicated in cell volume regulation. Expression of ClC-2 in Sf9 cells enhanced the rate of RVD [79] as well as in *Xenopus* oocytes [80] and delivery of a specific antibody against an essential regulatory domain of ClC-2 delayed RVD in rat HTC hepatoma cells that endogenously express ClC-2 [81]. However, $Clcn2^{-/-}$ mice did not show severe histological alterations in organs exposed to changes in osmolarity [82] and parotid acinar cells from $Clcn2^{-/-}$ mice recovered volume with similar efficiency to wild-type mice [83]. Since extracellular acidification significantly shifts ClC-2 activation to less hyperpolarized potentials resulting in the opening of ClC-2 at normal resting potential, it has been suggested that ClC-2 currents may counteract cell swelling in pathological conditions such as hypoxia [78].

ClC-3 has also been proposed to be the channel responsible for $I_{Cl,swell}$ [84,85]. In agreement with these studies, intracellular dialysis with an antibody against ClC-3 inhibited native volume-activated chloride currents in guinea pig cardiac cells and canine pulmonary arterial smooth muscle cells as well as a swelling-activated chloride current in NIH/3T3 cells transfected with the guinea pig ClC-3 gene [86]. Furthermore, ClC-3 antisense oligonucleotides have been shown to decrease an endogenous swelling-activated chloride current and the rate of RVD in bovine non-pigmented ciliary epithelial cell [87] as well as in HeLa cells and in *X. laevis* oocytes [88]. In contrast, disruption of the *Clcn3* gene in mice did not affect swelling-activated chloride current as tested in hepatocytes, pancreatic acinar cells [89] and salivary acinar cells [90].

In order to test the hypothesis of ClC-3 as the channel responsible for $I_{Cl,swell}$, we decided to generate HEK293 cell lines permanently expressing human ClC-3 (hClC-3) [91]. Both long and short versions of hClC-3, differing by 58 amino acids at the N-terminal, were tested. Using a combined approach of biochemistry and confocal microscopy, hClC-3 was shown to localize at the plasma membrane as well as in intracellular vesicles of the clones selected for over-expression of hClC-3-GFP . Whole cell patch clamp revealed that the magnitude of the $I_{Cl,swell}$ detected in clones expressing hClC-3 was not statistically different from parental HEK293 not expressing ClC-3. Furthermore, a HEK293 clone expressing hClC-3 bearing a mutation in position 579 (N579K), previously shown to abolish the outward rectification and change the selectivity of the $I_{Cl,swell}$ attributed to expression of ClC-3 [84], did not show either of the two mentioned changes. Finally, RVD was also not affected by the over-expression of hClC-3. Therefore,

these results show that hClC-3 is not the swelling-activated chloride channel involved in cell volume regulation. In addition to our own data, heterologous expression of the short version of rat ClC-3 in Chinese hamster ovary-K1 cells showed a current with strong outward rectification, lacking time inactivation at positive potentials, NPPB insensitive and with a selectivity $Cl^- > I^-$ [92]. This current was not swelling-activated and differed significantly from the endogenous swelling-activated NPBB-sensitive current but was very similar to the current recorded for expression of ClC-5, a channel highly homologous to ClC-3 [93]. Indeed, expressing ClC-3 bearing the mutation E224A, situated in a conserved sequence shown to line the permeation pathway of the ClC channels, generated a current largely inward rectifying and with reduced maximal conductance [94]. The identical mutation also changed channel conductance and rectification in ClC-4 and ClC-5 [93]. Taken together, these data show that ClC-3 is not activated by cell swelling, is not involved in RVD and that, in an over-expression system, its activity can be recorded on the cell membrane. Its biophysical characteristics are similar to the homologous channels ClC-4 and ClC-5 but differ significantly from the characteristics of the $I_{Cl,swell}$ current.

Transfection of the long version of hClC-3 has been shown to generate a calcium/calmodulin-dependent protein kinase II (CaMKII)-activated chloride current [95]. This current was not activated by cell swelling but elicited by introducing autonomously active CaMKII into the cell via the patch clamp pipette. Furthermore, raising the intracellular calcium concentration induced trafficking of vesicles containing long hClC-3 from the cytoplasm to the plasma membrane resulting in an increase of hClC-3 at the cell surface [95]. Since an increase in intracellular calcium upon cell swelling has been reported [16], we therefore tested whether elevation of intracellular calcium, by exposure to a calcium ionophore, could induce insertion and activation of short hClC-3 at the plasma membrane. Upon raising the intracellular calcium, by the application of 10 μM ionomycin, a calcium ionophore, we could not observe any translocation of intracellular vesicles containing hClC-3-GFP to the plasma membrane (Fig. 3, panel B). Furthermore, similar calcium-activated chloride currents were recorded after stimulation with the calcium ionophore A23187, in both the HEK293 parental cell line and the clone expressing short hClC-3 [91]. Ca-dependent chloride currents were also recorded in parotid acinar cells either from *Clcn3*$^{-/-}$ mice and wild-type mice [90]. Therefore, calcium seems not to be either a stimulus for the activation of ClC-3.

It has been shown that two kidney-specific members of the ClC family, ClC-Ka and ClC-Kb, target to the plasma membrane only in the presence of the β-subunit protein Barttin, in the presence of which they are constitutively open [96]. Furthermore, a splicing variant of long ClC-3, called ClC-3B, has been recently cloned [97]. ClC3B contains a motif thought to interact with the PDZ domain of the protein EBP50 (also called NHERF), which in turn is implicated in the localization of proteins such as Na^+–H^+ exchanger and CFTR to the epithelial apical membrane. The putative relocation of ClC-3B to the plasma membrane by EBP50 has been challenged by the finding that ClC-3B mainly localizes to the Golgi by interaction with the Golgi-associated protein GOPC [98]. It is well established, from functional [56] and structural studies [99,100] that ClC channels are homodimers and can form heterodimers with different characteristics from their respective homodimers [101]. For the above reasons, it is clear that targeting and localization of ClC-3 is rather

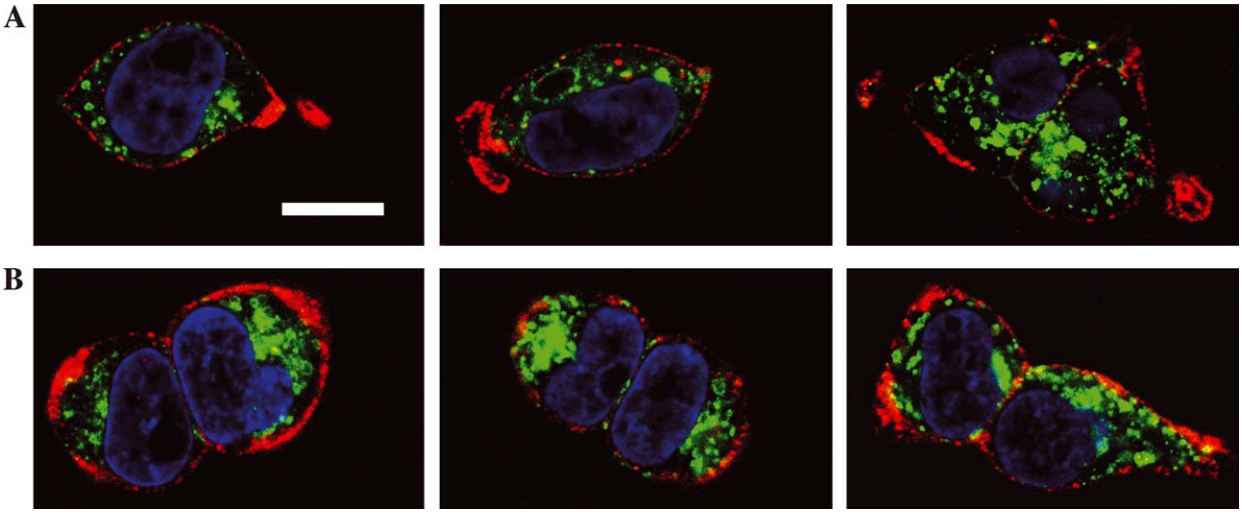

Fig. 3. Effect of increasing intracellular calcium concentration on the localization of short hClC-3-GFP. Single confocal sections of HEK293 cells permanently expressing short hClC-3-GFP (in green). In blue, Dapi staining of nuclear DNA, and in red, immunostaining of Na^+/K^+ ATPase at the plasma membrane with mouse anti-Na^+/K^+ ATPase α1 subunit antibody (clone C464.6, Research Diagnostics). Sections have been selected in order to show intracellular localization of short hClC-3-GFP so that a possible translocation and/or insertion into the plasma membrane can be easily detected. The plasma membrane is identified by the presence of Na^+/K^+ ATPase. Panel A: control; Panel B: cells that have been exposed for 20 min at 37 °C to 10 μM ionomycin in the presence of 10 mM $CaCl_2$ prior to fixation and processing. Dimensional bar represents 10 μm.

complex and may be modulated differently in different systems. Nonetheless, to quote David Clapham: "It does not seems likely that all the groups' data can be completely reconciled by proposing missing ancillary subunits or heteromultidimerization between ClC subtypes." [102].

In conclusion, ClC-3 seems to be a chloride channel similar in behaviour to the homologous ClC-4 and ClC-5 and not the elusive channel responsible for $I_{Cl,swell}$.

5. Concluding remarks

Why is it difficult to identify the channel responsible for $I_{Cl,swell}$? Functional cloning of $I_{Cl,swell}$ is hampered by several factors. (1) $I_{Cl,swell}$ is an ubiquitous current, therefore functional expression cloning requires detection over and above a significant background. (2) High affinity specific blockers of the current are not available. (3) The magnitude of the current, its rate of activation and its regulation are easily perturbed by exogenous and endogenous factors making quantitative analysis difficult.

Different approaches to the identification of the gene encoding $I_{Cl,swell}$ such as screening of genome-wide deletion mutant libraries [103] may be impossible if we assume that disrupting the gene coding for $I_{Cl,swell}$ results in a lethal phenotype, so that candidate genes might not be recognized. The use of simple organisms with a small fully sequenced genome like *C. elegans* may be of more use since gene expression can be suppressed by the use of siRNA technology and the resulting phenotype assessed more easily than in a complex mammalian system [104].

It seems therefore that we are still far from the identification of the gene/s coding for the protein/s responsible for $I_{Cl,swell}$ but new approaches have to be devised to pin down the molecular identity of $I_{Cl,swell}$.

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