

## Perspective

# Molecular Identity of the Outwardly Rectifying, Swelling-activated Anion Channel: Time to Reevaluate pICln

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Cells respond to swelling by activating anion and cation channels that allow the passive loss of inorganic ions and organic solutes. Net solute efflux accompanied by osmotically obliged water functions to return cell volume towards its original value, a process termed regulatory volume decrease.

An apparently ubiquitous response to swelling in vertebrate cells is activation of an outwardly rectifying anion current termed  $I_{Cl,swell}$ . The general characteristics of this current include an Eisenman type I anion permeability sequence ( $I^- > Br^- > Cl^- > F^-$ ), modest outward rectification, voltage-dependent inactivation at potentials above  $E_{Cl}$ , inhibition by a wide variety of compounds, including conventional anion transport inhibitors, and block by extracellular nucleotides such as ATP (reviewed by Strange et al., 1996; Okada, 1997). The degree of rectification, voltage sensitivity, and pharmacology can vary somewhat between different cell types. It is not clear whether the differences observed reflect the existence of distinct channels, or whether they are due to experimental and/or physiological variables. For example, channel voltage sensitivity is altered by intracellular  $Mg^{2+}$  concentration (Okada, 1997), the transmembrane  $Cl^-$  gradient, and by the presence of foreign anions in the extracellular solution (Meyer and Korbacher, 1996; Okada, 1997; my unpublished observations). In this article, I will assume for the sake of simplicity that  $I_{Cl,swell}$  arises from the activity of a single channel type or a family of closely related channels.

The channel responsible for  $I_{Cl,swell}$  has been referred to by a variety of names, including VRAC (volume regulated anion channel) and VSOR (volume expansion-sensing outwardly rectifying anion channel). Another name for the channel is VSOAC (volume-sensitive organic osmolyte/anion channel), which was coined to reflect the putative role it plays in the transport of organic anions and electroneutral organic osmolytes (Strange et al., 1996; Kirk and Strange, 1998). To avoid confusion, I will refer to the channel in this paper simply as the  $I_{Cl,swell}$  channel.

The route to the molecular identification of the  $I_{Cl,swell}$  channel has been an elusive and exceptionally

confusing one. The first purported molecular sighting of the channel was made in 1992. Valverde et al. (1992) and Gill et al. (1992) proposed that P-glycoprotein, the product of the multidrug resistance-1 gene, functions as both a drug transporter and the  $I_{Cl,swell}$  channel. However, numerous laboratories have been unable to reproduce the findings of these investigators and additional experimental observations have failed to support the original hypothesis (reviewed by Wine and Luckie, 1996; Okada, 1997). Because of this, the postulated role for P-glycoprotein as a volume-activated anion channel is no longer considered to be a tenable idea by most workers in the field.

More recently, it has been suggested that P-glycoprotein functions to modulate or regulate  $I_{Cl,swell}$  (reviewed by Wine and Luckie, 1996; Okada, 1997). As discussed by Wine and Luckie (1996), the channel regulator concept may be stretched too far in the case of P-glycoprotein. It is not clear whether the apparent modulation of  $I_{Cl,swell}$  by P-glycoprotein reflects a physiologically relevant function, or whether it is simply a consequence of overexpression of the protein induced by transfection or drug selection.

A second purported sighting of the  $I_{Cl,swell}$  channel was also made in 1992. Paulmichl et al. (1992) reported the expression cloning of a cDNA encoding a protein termed pICln. When expressed in *Xenopus* oocytes, pICln induced an outwardly rectifying anion conductance that was blocked by extracellular nucleotides and inactivated by depolarizing voltages. Although the pICln-associated current did not require swelling for activation, its characteristics resembled, at least superficially, those of  $I_{Cl,swell}$ .

Paulmichl et al. (1992) proposed initially that pICln is an anion channel-forming protein with a novel structure. Hydrophobicity analysis indicated that pICln lacks transmembrane helices believed to form the pore of most vertebrate ion channels. It was instead proposed that the pICln "channel" was a homodimer, and that each monomer contained four  $\beta$  strands that contribute to the formation of an eight-stranded, antiparallel  $\beta$  barrel transmembrane pore. The putative  $\beta$  barrel

pore structure is reminiscent of that of bacterial and mitochondrial porins (Jap and Walian, 1996).

Compelling evidence was presented to support the hypothesis that pICln is a channel-forming protein. A possible nucleotide binding site was identified in the second  $\beta$  strand of each pICln monomer. This site has poor homology with known nucleotide binding motifs (see Saraste et al., 1990), but its location within the putative channel pore was consistent with the inhibition by extracellular nucleotides of the pICln-associated current (Paulmichl et al., 1992). Mutation of the binding site resulted in the apparent expression of an anion current that was no longer inhibited by nucleotides. The "mutant" current also had altered voltage sensitivity and, unlike the current induced by wild-type pICln, was inhibited by removal of extracellular  $\text{Ca}^{2+}$  (Paulmichl et al., 1992).

Shortly after the publication of these studies, Krapivinsky et al. (1994) proposed that pICln was not a channel, but was instead an anion channel regulator. Four pieces of indirect evidence were presented to support this idea. First, biochemical and immunofluorescence studies demonstrated that the protein was localized primarily in the cytoplasm rather than the plasma membrane as expected for an ion channel. Second, oocyte swelling was shown to activate an endogenous  $I_{\text{Cl,swell}}$  (see also Ackerman et al., 1994) that superficially resembled the current induced by heterologous expression of pICln. This observation led the authors to conclude incorrectly (discussed below) that the current induced by expression of pICln was the same as that induced by oocyte swelling. Third, oocytes express a pICln homolog, and fourth, microinjection of an anti-pICln monoclonal antibody into the oocyte cytoplasm inhibited swelling-induced activation of the endogenous  $I_{\text{Cl,swell}}$  slowly over a period of 8–20 h. These latter two observations suggested that pICln was critical to the function of the  $I_{\text{Cl,swell}}$  channel (Krapivinsky et al., 1994). Since pICln did not appear to be present in the plasma membrane, the authors concluded that the protein was a regulator of the  $I_{\text{Cl,swell}}$  channel, and that heterologous overexpression of the protein activated the channel in the absence of cell swelling. While intriguing, this hypothesis was troubling to many workers in the field because it did not take into account the results of mutagenesis studies described by the authors in their first report on pICln (Paulmichl et al., 1992). Thus, we proposed an alternate hypothesis in which pICln is a channel activated by reversible insertion into the plasma membrane (Strange et al., 1996).

The controversy over whether pICln forms a transmembrane transport pathway, or whether it is a transport regulator, is similar to the controversies regarding the functions of other transport proteins (see Tzounopoulos et al., 1995; Shimbo et al., 1995). To investi-

gators interested in cellular osmoregulation, the cloning of any protein, be it a volume-sensitive channel or transporter, or accessory/regulatory molecule involved in volume homeostasis, is interesting and important because it provides a new tool to explore the ever-vexing and still unsolved puzzle of how cells sense volume changes and transduce those signals into regulatory responses. However, recent work by Buyse et al. (1997), as well as my laboratory, have suggested that, like P-glycoprotein, the postulated pICln- $I_{\text{Cl,swell}}$  channel connection may be leading the field in the wrong direction.

Voets et al. (1996) recently compared the characteristics of the current induced by expression of human pICln in *Xenopus* oocytes to the endogenous  $I_{\text{Cl,swell}}$ . While the currents superficially resemble one another, there are very clear differences when one looks closely (Table I). The pICln-induced current is much more strongly rectified than  $I_{\text{Cl,swell}}$ , has a different anion permeability sequence, and is not activated by cell swelling. Cyclamate permeates the  $I_{\text{Cl,swell}}$  channel, but blocks the channel responsible for the pICln-induced current. cAMP blocks both channels, but the block of the  $I_{\text{Cl,swell}}$  channel is voltage independent, while the block of the pICln-induced channel only occurs with depolarization above the reversal potential. Finally, both currents exhibit depolarization-induced inactivation, but inactivation of the  $I_{\text{Cl,swell}}$  channel is increased by elevation of extracellular pH.

Taken together, these observations cast some doubt on the hypothesis that pICln is an anion channel regulator (Krapivinsky et al., 1994). If expression of pICln is simply turning on an endogenous  $I_{\text{Cl,swell}}$  channel, why should the characteristics of the current induced by swelling and pICln cRNA injection differ? One might argue that species differences, different activation modes (i.e., swelling versus heterologous protein expression), or other factors account for this disparity. However, such arguments seem moot in light of the demonstration by Buyse et al. (1997) that (a) expression of an un-

TABLE I  
Comparison of  $I_{\text{Cl,swell}}$  and pICln-induced Current (ICln) in *Xenopus* oocytes (from Voets et al., 1996)

Current	$I_{\text{Cl,swell}}$	ICln
Rectification ratio*	1.46	4.34
Anion permeability	$\text{I}^- \geq \text{NO}_3^- \geq \text{Cl}^-$	$\text{NO}_3^- > \text{I}^- > \text{Cl}^-$
Effect of extracellular cyclamate	Low permeability	Channel block
Block by extracellular cAMP	Voltage independent	Voltage dependent
Voltage-dependent inactivation	pH sensitive	pH insensitive
Effect of cell swelling	Activation	No effect

\*Rectification ratio is the ratio of currents measured at +55 and -95 mV.

related protein, ClC-6, induces the same current as that induced by expression of pICln, and (b) the pICln-associated current is observed in ~5–6% of uninjected control oocytes (see also Paulmichl et al., 1992). The simplest conclusion from these findings is that expression of certain heterologous proteins “activates” an endogenous, outwardly rectifying anion current that is distinct from the endogenous  $I_{Cl,swell}$ . This is not an unprecedented finding. Previous studies have shown that endogenous oocyte currents are activated by expression of a variety of heterologous proteins (Tzounopoulos et al., 1995; Shimbo et al., 1995).

If pICln and apparently unrelated proteins are simply activating an endogenous oocyte conductance, how can the pICln mutagenesis experiments described by Paulmichl et al. (1992) be explained? Recent evidence indicates that the current ascribed to expression of the pICln nucleotide binding site mutant is also due to the activity of an endogenous oocyte anion channel, possibly the well-described  $Ca^{2+}$ -activated  $Cl^-$  channel (e.g., Wu and Hamill, 1992). Buyse et al. (1997) have reported that uninjected oocytes express a current with characteristics identical to those of the current associated with expression of the mutant pICln. In addition, oocyte expression of the so-called “AAA” nucleotide binding site mutant (Paulmichl et al., 1992), which has all three glycine residues comprising the putative nucleotide binding site mutated to alanine residues, induced a conductance with characteristics identical to that induced by wild-type pICln (Voets et al., 1998). These characteristics include block by extracellular nucleotides, insensitivity to extracellular  $Ca^{2+}$ , and inactivation at positive membrane potentials.

These observations indicate that the currents ascribed to the expression of pICln very likely arise from endogenous oocyte anion channels that are distinct from the  $I_{Cl,swell}$  channel. How can these results be reconciled with observations suggesting that there is a direct link between pICln and  $I_{Cl,swell}$ ? In my opinion, there is no obvious way to do so. Instead, a critical re-evaluation of the data indicates that none of it provides a compelling basis for thinking that pICln plays a role in volume homeostasis.

Krapivinsky et al. (1994) proposed the regulator hypothesis in part because most of the pICln appeared to be in the cytoplasm rather than localized to the plasma membrane. Other investigators have confirmed this finding. Buyse et al. (1997), using membrane fractionation techniques and confocal immunofluorescence microscopy, demonstrated a largely cytoplasmic location in control and swollen endothelial cells. We have made similar observations in C6 glioma cells. In addition, we were unable to detect membrane localization of pICln in either control or swollen C6 cells that had been transfected with carboxy- or amino-terminus green fluo-

rescent protein (GFP)-pICln fusion constructs (Emma et al., 1998).

In contrast to these findings, studies carried out in other laboratories have reported an apparent membrane localization of pICln. Schwartz et al. (1997) concluded that pICln was “concentrated in the membrane” of red blood cells. However, the low resolution, confocal immunofluorescence images presented in their paper do not allow an accurate assessment of whether the protein is truly localized to the membrane or whether it is associated with submembranous cytoskeleton, as their biochemical studies suggest (discussed below). Fractionation studies carried out on MDCK cells (Laich et al., 1996), embryonic skate heart cells (Musch et al., 1997), and neonatal rat myocytes (Goldstein et al., 1997) have revealed that swelling induces an increase of pICln content in the particulate or membrane fraction. However, such findings must be interpreted cautiously. They certainly do not demonstrate that pICln inserts into the plasma membrane in response to swelling. The apparent swelling-induced association with the particulate fraction may represent an association of pICln with membrane-attached cytoskeleton rather than with the lipid bilayer. Immunoprecipitation (Krapivinsky et al., 1994; Sanchez-Olea et al., 1997), *in vitro* protein binding assays (Krapivinsky et al., 1994; Sanchez-Olea et al., 1997), and yeast two-hybrid studies (Schwartz et al., 1997) have indicated that pICln interacts with cytoskeletal components including actin and the nonmuscle isoform of the alkali myosin light chain. pICln can be readily extracted from red cell ghost membranes by treatment with low ionic strength buffers, which also extract the spectrin-actin cytoskeleton (Schwartz et al., 1997).

If the apparent swelling-induced association of pICln with the membrane fraction represents an interaction with the cytoskeleton, does this imply that the protein is playing some regulatory role in volume homeostasis? Not necessarily. Numerous studies have shown that cell swelling alters the structure of the cytoskeleton, particularly F-actin (reviewed by Okada, 1997). Thus, changes in the interaction of pICln with the cytoskeleton may reflect events that are not directly related to cell volume homeostasis.

The ability to reconstitute, from purified pICln, anion channel activity with the characteristics of the  $I_{Cl,swell}$  channel would provide important support for the pICln channel hypothesis. We have now carried out extensive planar lipid bilayer reconstitution studies of recombinant pICln and have been unable to detect anion channel activity with this protein (Li et al., 1998). However, we have consistently observed a highly cation-selective channel in our bilayer experiments. Reconstitution of pICln in liposomes increases  $^{86}Rb$  flux but has no effect on  $^{36}Cl$  transport, suggesting strongly that the

cation channel activity we see is due to pICln rather than contaminant proteins. It must be stressed here that the channel activity we observe in bilayers does not imply that pICln functions in vivo as a cation channel. Many proteins that are clearly not ion channels give rise to channel-like activity when reconstituted into artificial membranes.

As noted earlier, injection of monoclonal anti-pICln antibodies into *Xenopus* oocytes inhibited activation of the endogenous  $I_{Cl,swell}$  (Krapivinsky et al., 1994). This inhibition occurred slowly over a period of 8–20 h. While this effect is intriguing, it does not provide definitive or even compelling proof of a pICln– $I_{Cl,swell}$  channel connection. Whatever pICln is, it is probably important to cell function. The protein is ubiquitous, abundant, and has a structure that is highly conserved among evolutionarily divergent species (e.g., Krapivinsky et al., 1994). The effects of long-term disruption of pICln function on cell physiology are unknown. Activation of  $I_{Cl,swell}$  can be dramatically reduced by a variety of seemingly unrelated parameters such as decreases in cellular ATP levels (Strange et al., 1996; Okada, 1997), increases in cytoplasmic ionic strength (Emma et al., 1997) and  $Mg^{2+}$  concentration (Okada, 1997), dissociation of cells from their growth substrate (Han et al., 1996), disruption of the cytoskeleton (Levitan et al., 1995; Zhang et al., 1997), and unidentified “environmental factors” (Jackson et al., 1996). The ability of defolliculated oocytes to respond to swelling by activation of  $I_{Cl,swell}$  declines slowly over a period of days when the cells are maintained in relatively simple salt solutions (Ackerman et al., 1994; Hand et al., 1997). Many of the factors that inhibit  $I_{Cl,swell}$  do not prevent activation per se, but instead simply shift the volume set-point of the channel such that greater degrees of swelling are required to activate it (Emma et al., 1997; Basavappa and Strange, 1998). Taken together, these observations suggest that the inhibitory effect of anti-pICln on  $I_{Cl,swell}$  could be a very indirect one. For example, one might postulate that pICln is a protein involved in maintaining and regulating cytoskeletal function, and that experimental perturbation of pICln alters cytoskeletal architecture. Since disruption of the cytoskeleton alters the volume sensitivity of  $I_{Cl,swell}$  (Levitan et al., 1995; Zhang et al., 1997), concluding that pICln is a “regulator” of the channel is no more appropriate than concluding that drugs such as phalloidin and cytochalasin are channel regulators. The studies of Gschwendner et al. (1995), which demonstrated an ~40% inhibition of  $I_{Cl,swell}$  in pICln antisense-transfected fibroblasts, are subject to the same criticisms.

Hubert et al. (1998) have shown recently that transfection of pICln into tsA201a cells increases the rate of  $I_{Cl,swell}$  activation 16-fold. Conversely, transfection with pICln antisense decreases the rate of current activation.

These are interesting findings, but ones that must again be viewed cautiously. Do these results indicate that pICln is regulating  $I_{Cl,swell}$ , or do changes in its expression affect swelling-induced current activation through indirect mechanisms such as alterations in cytoskeletal structure?

#### CONCLUSIONS AND PERSPECTIVE

There is some evidence to suggest a link between pICln and the  $I_{Cl,swell}$  channel, but this evidence is neither definitive nor compelling. More importantly, it is difficult to reconcile findings that argue for a pICln– $I_{Cl,swell}$  channel link with studies suggesting that the results of initial investigations on pICln (Paulmichl et al., 1992) were artifacts related to the use of the oocyte expression system (Buyse et al., 1997; Voets et al., 1997). The central question regarding pICln has shifted from a debate over whether the protein is a swelling-activated anion channel or channel regulator, to a debate about whether it has anything at all to do with  $I_{Cl,swell}$ . pICln may have functions related to cell volume homeostasis, ion channels, and membrane transport processes, but these may be quite indirect.

Identification of the protein(s) responsible for  $I_{Cl,swell}$  will continue to be a very difficult and challenging problem. There are two reasons for this. First, expression cloning and characterization of heterologously expressed ion channels requires a cell system with minimal or no background expression of the channel of interest.  $I_{Cl,swell}$  is expressed ubiquitously in mammalian cells and is also present in *Xenopus* oocytes (Ackerman et al., 1994; Hand et al., 1997). Cells types such as insect Sf9 cells may lack  $I_{Cl,swell}$ , but they possess other swelling-activated anion currents (C.E. Bear, personal communication) that will confound expression studies. Second, the native  $I_{Cl,swell}$  channel has only recently come under detailed study. Our understanding of its biophysical characteristics is therefore far from complete. Importantly, despite intense efforts, we know almost nothing about how  $I_{Cl,swell}$  is activated by swelling. Without such detailed knowledge, the chances for false starts and confusion are increased when molecular candidates for the channel itself or channel regulators are put forth. Clearly, much additional rigorous cellular and biophysical characterization of  $I_{Cl,swell}$  is needed and warranted.

Currently, there are, in my opinion, only two molecular candidates for volume regulated anion channels that are backed up by persuasive experimental evidence, ClC-2 and ClC-3. ClC-2 was shown to be swelling-activated by expression in *Xenopus* oocytes (Grunder et al., 1992; Jordt and Jentsch, 1997). cRNA injections were performed on defolliculated oocytes that were maintained in simple salt solutions, conditions that dra-

matically suppress the endogenous  $I_{Cl,swell}$  (see Ackerman et al., 1994; Hand et al., 1997). The characteristics of ClC-2 current are substantially different from  $I_{Cl,swell}$ , indicating that it is unlikely to be the  $I_{Cl,swell}$  channel. Furthermore, the role of ClC-2 in volume homeostasis is uncertain since similar swelling-activated currents are not readily observable in mammalian cells (reviewed by Strange et al., 1996; see Carew and Thorn, 1996, for an exception to this generalization). Nevertheless, studies of volume-dependent gating of ClC-2 may provide important clues about  $I_{Cl,swell}$  regulation and the more general problem of how cells sense volume changes.

ClC-3 cloned from guinea pig heart gives rise to an outwardly rectifying anion current that is activated by cell swelling when it is expressed in NIH/3T3 cells (Duan et al., 1997). Experiments on ClC-3 were performed using a slow rate of cell swelling and a patch pipette solution with high ionic strength. Both of these maneuvers will reduce the rate of  $I_{Cl,swell}$  activation

(Emma et al., 1997; Basavappa and Strange, 1998). The ClC-3 current has characteristics remarkably similar to those of  $I_{Cl,swell}$  with one major exception—ClC-3 is inhibited by activation of protein kinase C (Duan et al., 1997; Kawasaki et al., 1994). In rabbit atrial myocytes,  $I_{Cl,swell}$  is also inhibited by PKC activation (Duan et al., 1995). This is very different from  $I_{Cl,swell}$  in other cells where PKC has no inhibitory effect (e.g., Szűcs et al., 1996; reviewed by Okada, 1997), and may actually stimulate channel activity (Jackson and Strange, 1993). The findings of Duan et al. (1997) need to be reproduced and confirmed with additional studies. If they can be, it will be interesting and important to determine if a non-PKC-regulated ClC-3 isoform, a ClC-3 heteromultimer (Lorenz et al., 1996), or another member of the ClC family accounts for  $I_{Cl,swell}$  in other cells types. Given the confusion that exists over the molecular nature of  $I_{Cl,swell}$ , it will be important to subject this hypothesis to cautious and rigorous scrutiny.

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