The chloride current induced by expression of the protein pI_{Cln} in *Xenopus* oocytes differs from the endogenous volume-sensitive chloride current

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1. Phenotypical similarities between $I_{Cl,swell}$, the cell-swelling-induced chloride current and I_{Cln}, the nucleotide-sensitive chloride current induced by expression of mammalian pI_{Cln} in *Xenopus* oocytes, have led to models which identify pI_{Cln} either as the volume-sensitive chloride channel or as a cytosolic regulator thereof.

2. To investigate critically the relationship between $I_{Cl,swell}$ and pI_{Cln} two-microelectrode voltage clamp experiments were performed on *Xenopus* oocytes in which either human pI_{Cln} was expressed or endogenous $I_{Cl,swell}$ was activated .

3. Several criteria that clearly differentiated I_{Cln} from $I_{Cl,swell}$ were detected. Outward rectification and the discrimination between NO_3^- and Cl^- were more pronounced for I_{Cln}. Cyclamate blocked I_{Cln} but not $I_{Cl,swell}$. In contrast to $I_{Cl,swell}$, inactivation kinetics of I_{Cln} were pH independent and extracellular cAMP blocked only the outward I_{Cln} component. Finally, I_{Cln} was readily expressed in collagenase-defolliculated oocytes and was not modulated by extracellular hypotonicity, whereas $I_{Cl,swell}$ could only be triggered in follicle-enclosed or manually defolliculated oocytes.

4. We therefore conclude that I_{Cln} and $I_{Cl,swell}$ are two different chloride currents. Consequently, any model which invokes a crucial role for pI_{Cln} in $I_{Cl,swell}$ should be critically reviewed.

Increases in cell volume induced by extracellular hypotonicity or intracellular accumulation of osmolytes activate a chloride current, $I_{Cl,swell}$, that is ubiquitously present in both mammalian and non-mammalian cells. Efflux of chloride ions via $I_{Cl,swell}$ contributes to the mechanism of regulatory volume decrease which restores the cellular volume to its original value (Baumgarten & Feher, 1995). The biophysical and pharmacological properties of $I_{Cl,swell}$ have been extensively characterized (for a review see Nilius, Eggermont, Voets & Droogmans, 1996; Strange, Emma & Jackson, 1996). However, the molecular identity of $I_{Cl,swell}$, as well as the molecular components of its activation cascade, is still unknown.

One protein that has been implicated in $I_{Cl,swell}$ is pI_{Cln}. This protein was originally cloned from a Madin–Darby canine kidney (MDCK) cDNA library through its ability to induce a nucleotide-sensitive chloride current, I_{Cln}, when expressed in *Xenopus* oocytes (Paulmichl, Li, Wickman, Ackerman, Peralta & Clapham, 1992). I_{Cln} is an outwardly rectifying, anion-selective ($I^- > Br^- > Cl^-$) current that slowly inactivates at positive potentials. Because of their comparable phenotypes, I_{Cln} has been equated to $I_{Cl,swell}$ (Krapivinsky, Ackerman, Gordon, Krapivinsky & Clapham, 1994; Gschwentner, Nagl, Wöll, Schmarda, Ritter & Paulmichl, 1995; Strange *et al.* 1996). At the molecular level, it has been proposed that pI_{Cln} functions as the volume-activated channel itself (Gschwentner *et al.* 1995; Strange *et al.* 1996) or as a cytosolic or membrane-tethered regulator of the channel (Krapivinsky *et al.* 1994; Coca-Prados, Sánchez-Torres, Peterson-Yantorno & Civan, 1996). Functional data supporting the link between pI_{Cln} and $I_{Cl,swell}$ have also been obtained. Injection of a monoclonal antibody against pI_{Cln} in *Xenopus* oocytes suppressed the endogenous $I_{Cl,swell}$ (Krapivinsky *et al.* 1994). Furthermore, antisense oligonucleotides against pI_{Cln} partially reduced $I_{Cl,swell}$ in NIH 3T3 fibroblasts (Gschwentner *et al.* 1995).

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We have previously isolated cDNA clones coding for human pI_{Cln} (Buyse, De Greef, Raeymaekers, Droogmans, Nilius & Eggermont, 1996). We have now expressed human pI_{Cln} in *Xenopus* oocytes in order to examine critically the relationship between I_{Cln} and the endogenous $I_{Cl,swell}$. A systematic comparison between I_{Cln} and $I_{Cl,swell}$ in *Xenopus* oocytes reveals that these two currents can be clearly discriminated by biophysical, pharmacological and biological criteria. We therefore conclude that I_{Cln} and $I_{Cl,swell}$ are two different currents and we question the previously proposed tight link between pI_{Cln} and $I_{Cl,swell}$.

METHODS

RNA preparation and injection in *Xenopus* oocytes

A human cDNA clone for pI_{Cln} (accession number X91788; Buyse *et al.* 1996) was polymerase chain reaction-mutagenized by replacing the 5′ untranslated region with an *Eco*RI–*Hind*III–*Bam*HI linker. This allowed subcloning of human pI_{Cln} cDNA as a *Bam*HI fragment in pGEMHE (Liman, Tytgat & Hess, 1992) yielding the pGEMHE–EHBhI_{Cln}ORF vector. The pGEMHE–EHBhI_{Cln}ORF vector was cut with *Hind*III. The linearized DNA was purified (QIAEX desalting and concentration protocol; Qiagen, Hilden, Germany) and *in vitro* transcribed with T7 RNA polymerase. pI_{Cln} RNA was extracted with phenol–chloroform, ethanol precipitated and resuspended in sterile RNAse-free H_2O.

For expression of I_{Cln}, stage V–VI *Xenopus* oocytes were isolated by partial ovariectomy under anaesthesia (tricaine, 1 g l^{-1}). Anaesthetized animals were then kept on ice during dissection. The oocytes were defolliculated by treatment with 2 mg ml^{-1} collagenase (Boehringer) in zero calcium ND-96 solution. Standard ND-96 contained (mM): 96 NaCl, 2 KCl, 1·8 $CaCl_2$, 1 $MgCl_2$, 5 Hepes, pH 7·5. Between 2 to 4 h after defolliculation, oocytes were injected with 50 nl of 1–100 ng μl^{-1} human pI_{Cln} RNA. The oocytes were then incubated at 18 °C for 2–4 days in ND-96 solution supplemented with gentamicin sulphate (50 mg ml^{-1}). To measure $I_{Cl,swell}$, we used either manually defolliculated oocytes or follicle-enclosed oocytes prepared by dissection from an ovarian lobe as described by Arrellano & Miledi (1995). The oocytes were then incubated in gentamicin sulphate-supplemented ND-96 at 18 °C and currents were recorded within 48 h of oocyte isolation.

Electrophysiological analysis

Whole-cell currents from oocytes were recorded using the two-microelectrode voltage clamp technique by means of a home-made amplifier. Resistances of voltage and current electrodes filled with 3 M KCl were 0·5–2 MΩ. Current records were sampled at 500 or 2000 μs intervals and filtered at 1 or 0·1 kHz, respectively, using a 4-pole low-pass Bessel filter. To eliminate the effect of voltage drop across the bath grounding electrode, the bath potential was actively controlled. Linear components of capacity and leak currents were not subtracted.

The standard bath solution was ND-96. NaCl (96 mM) was replaced with various sodium salts (I^-, NO_3^-, gluconate or cyclamate all at 96 mM) to obtain the anion permeability sequence. These experiments were performed using an agar bridge. Extracellular tonicity was reduced in two steps. First, we perfused the oocytes with isotonic ND-48–mannitol containing (mM): 48 NaCl, 2 KCl, 1·8 $CaCl_2$, 1 $MgCl_2$, 5 Hepes (pH 7·5) and 100 mannitol. After stabilization of the membrane current we switched to hypotonic ND-48 without mannitol. Anion permeability of hypotonicity-induced currents was examined by replacing NaCl (48 mM) with various sodium salts (48 mM). Three voltage protocols were used during the analysis. (i) Step protocol 1: from a holding potential of −20 mV, oocytes were clamped for 800 ms from −100 to +100 mV in 20 mV increments. (ii) Step protocol 2: oocytes were held at −70 mV and a 800 ms pulse to +80 mV was applied. (iii) Linear voltage ramp protocol: oocytes were held at −20 mV and a linear voltage ramp from −100 to +100 mV (0·4 V s^{-1}) was applied. All measurements were performed at 22–25 °C.

Permeability (P) ratios (P_X/P_{Cl}) for various anions (X) relative to Cl^- were calculated using the formula:

$$P_X/P_{Cl} = ([Cl]_o \exp(\Delta EF/RT) - [Cl]_{rest})/[X]_o,$$

where ΔE is the shift in reversal potential, $[Cl]_o$ is the extracellular Cl^- concentration in ND-96 or ND-48, $[X]_o$ is the extracellular anion concentration in anion-substituted ND-96 or ND-48, and $[Cl]_{rest}$ is the remaining Cl^- concentration in the anion-substituted media. Numerical data are represented as means ± S.E.M.

RESULTS

Expression of I_{Cln} and activation of $I_{Cl,swell}$ in *Xenopus* oocytes

Injection of human pI_{Cln} RNA in collagenase-defolliculated *Xenopus* oocytes resulted in an outwardly rectifying current that slowly inactivated at positive potentials (≥+60 mV) and that reversed between −25 and −35 mV in ND-96 (Fig. 1*A*). Extracellular anion substitutions affected the reversal potential as well as the current amplitude indicating that anions were the permeating substances (Fig. 2*A*). These properties correspond to the I_{Cln} phenotype that has been observed after expression of other mammalian pI_{Cln} proteins in *Xenopus* oocytes (Paulmichl *et al.* 1992; Abe, Takeuchi, Ishii & Abe, 1993; Okada, Ishii, Nunoki & Taira, 1995). We also tested whether I_{Cln} was modulated by changes in extracellular tonicity. When I_{Cln}-expressing *Xenopus* oocytes were perfused with isotonic ND-48–mannitol solution, we noticed a decrease in the outward current due to the reduction of the extracellular Cl^- concentration. Subsequent reduction of the extracellular tonicity by omitting mannitol for up to 20 min did not change the I_{Cln} phenotype in any aspect (Fig. 1*C*).

We then tried to activate the endogenous $I_{Cl,swell}$ in *Xenopus* oocytes. All attempts to trigger $I_{Cl,swell}$ in collagenase-defolliculated oocytes were unsuccessful. However, when manually defolliculated or follicle-enclosed oocytes were subjected to a hypotonic stimulus, we observed an outwardly rectifying current with slow inactivation kinetics at positive potentials within 5 min of switching to the hypotonic solution (Fig. 1*B* and *D*). Extracellular anion substitution indicated that the $I_{Cl,swell}$ channel discriminated poorly between anions (Fig. 2*B*). It has been described in detail elsewhere that this current is carried by Cl^- ions (Ackerman, Wickman & Clapham, 1994).

Being able to evoke both I_{Cln} and $I_{Cl,swell}$ in *Xenopus* oocytes we started to compare these currents in closer detail. One difference between these currents can be inferred from their current–voltage (I–V) relationships (Fig. 1*C* and *D*).

The I–V plot for both currents deviated from linearity, but the outward rectification pattern was significantly more pronounced for I_{Cln} than for $I_{Cl,swell}$. To quantify the degree of outward rectification, we calculated the ratio of the current at +55 mV over the current at −95 mV, i.e. at potentials that were approximately equidistant from the reversal potential. Current amplitudes were taken from the voltage ramp curves obtained in ND-48 so that the extracellular Cl^- concentration was identical for both currents. A rectification score of $1{\cdot}46 \pm 0{\cdot}11$ (number of oocytes, $n = 12$) was obtained for $I_{Cl,swell}$ whereas this value was $4{\cdot}34 \pm 0{\cdot}47$ ($n = 5$) for I_{Cln}.

I_{Cln} and $I_{Cl,swell}$ have a different anion permeability sequence

We then compared the anion permeability sequence of both currents. As reported by Ackerman *et al.* (1994) $I_{Cl,swell}$ discriminated poorly between NO_3^-, I^- and Cl^- as permeating anions (Fig. 2*B*). In contrast, we observed a significant permeability difference between NO_3^- and Cl^- with I_{Cln} (Fig. 2*A*). The shifts in reversal potential in the anion-substituted media were used to calculate permeability ratios (P_X/P_{Cl}). For I_{Cln} this yielded the following P_X/P_{Cl} values ($n = 6$): $1{\cdot}35 \pm 0{\cdot}04$ (NO_3^-); $1{\cdot}19 \pm 0{\cdot}02$ (I^-); $0{\cdot}64 \pm 0{\cdot}05$ (gluconate). Cyclamate acted as a channel blocker as

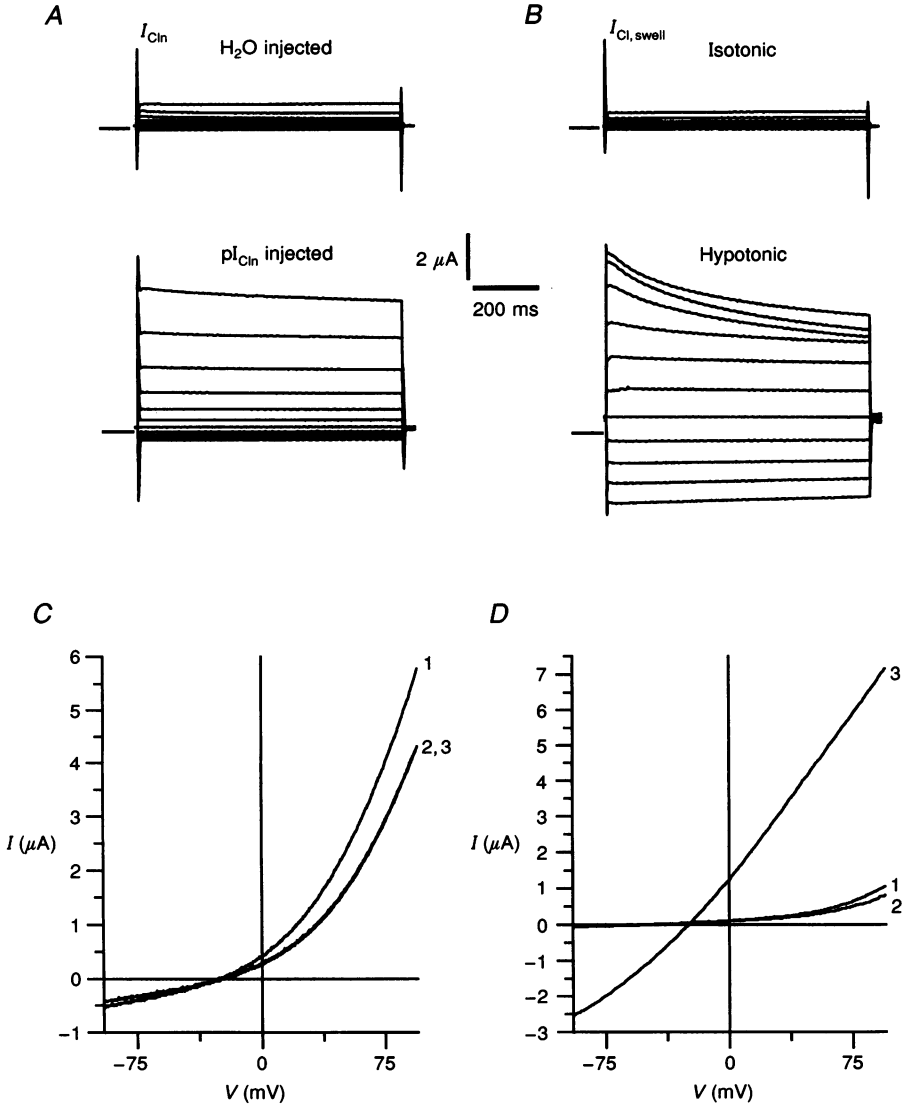

Figure 1. I_{Cln} and $I_{Cl,swell}$ expressed in *Xenopus* oocytes

A, collagenase-defolliculated *Xenopus* oocytes injected with H_2O (top) or human pI$_{Cln}$ RNA (bottom) were tested with step protocol 1 (see Methods) in ND-96. *B*, a manually defolliculated *Xenopus* oocyte was subjected to step protocol 1 in isotonic ND-96 solution (top) or after 5 min in hypotonic ND-48 solution (bottom). *C* and *D*, I–V relationships measured during the ramp protocol in ND-96 (trace 1), isotonic ND-48 (trace 2) or hypotonic ND-48 (trace 3) from a collagenase-defolliculated, pI$_{Cln}$-injected oocyte (*C*) or a manually defolliculated oocyte (*D*). The currents described in panels *A*–*D* are representative examples ($n \geqslant 5$).

can be deduced from the reduction of the inward current by cyclamate and by the leftward shift of the reversal potential (Fig. 2*C*). P_X/P_{Cl} values were also calculated for $I_{Cl,swell}$ ($n = 4$ or 5): 1·09 ± 0·02 (I^-); 1·06 ± 0·01 (NO_3^-); 0·70 ± 0·04 (gluconate); 0·61 ± 0·05 (cyclamate). Importantly, cyclamate was a permeating anion for $I_{Cl,swell}$ (Fig. 2*D*), which contrasts with its blocking effect on I_{Cln}. The differences in anion selectivity between both currents can be appreciated from the plot of their permeability ratios (Fig. 2*E*). A similar anion selectivity for I_{Cln} and $I_{Cl,swell}$ was obtained when relative current ratios (I_X/I_{Cl}) at +70 mV were calculated (data not shown). For $I_{Cl,swell}$ our I_X/I_{Cl} values corresponded to those reported by Ackerman *et al.* (1994).

I_{Cln} and $I_{Cl,swell}$ differ in the voltage dependency of the block by extracellular cAMP

A hallmark of I_{Cln} is its inhibition by extracellular nucleotides such as 5 mM cAMP (Paulmichl *et al.* 1992). Interestingly, we observed that the block by cAMP was voltage dependent (Fig. 3*A*). cAMP (5 mM) blocked 42 ± 7% of the current at

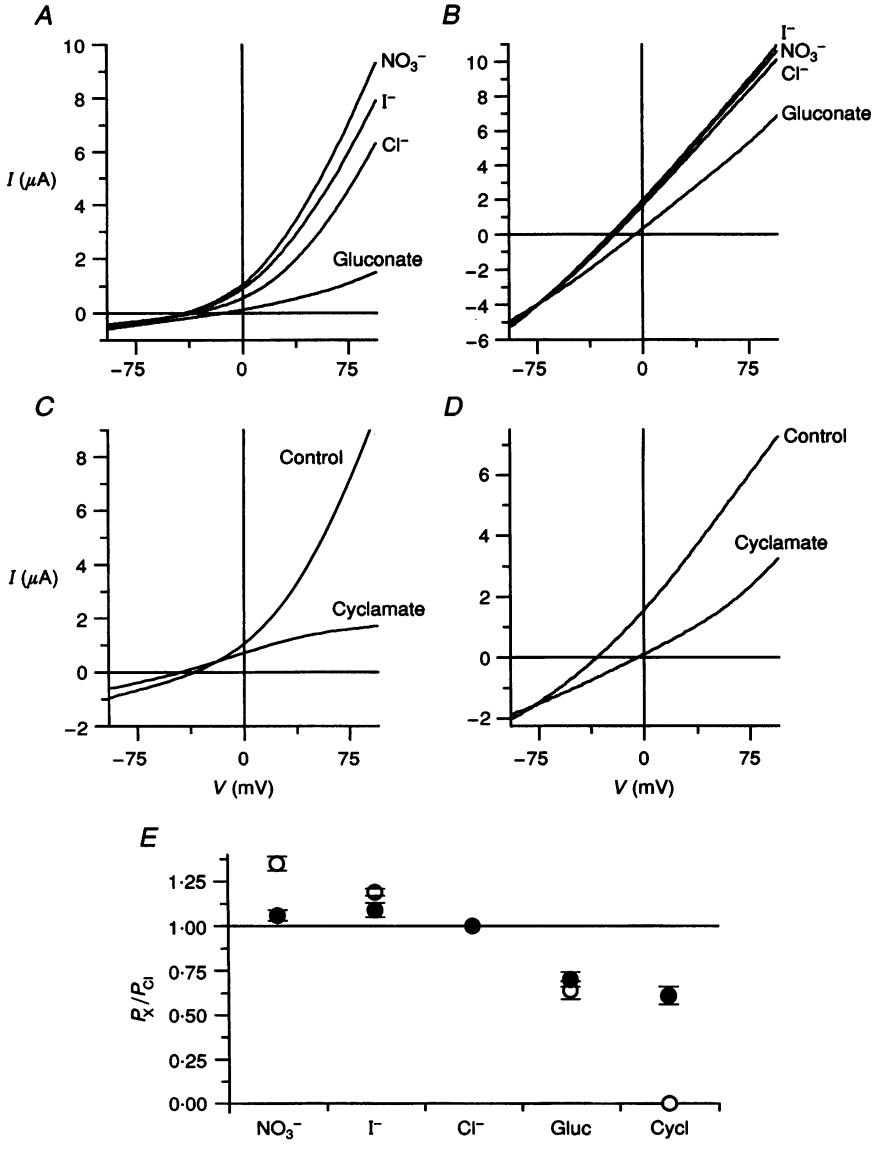

Figure 2. Anion selectivity of I_{Cln} and $I_{Cl,swell}$

A and *C*, *I–V* relationships measured during the ramp protocol from a pI_{Cln}-injected oocyte in standard ND-96 solution or anion-substituted ND-96 (96 mM NaI, $NaNO_3$, sodium gluconate (*A*) or sodium cyclamate (*C*) instead of NaCl). *B* and *D*, *I–V* relationships measured from a manually defolliculated oocyte in hypotonic ND-48 or in anion-substituted ND-48 (48 mM NaI, $NaNO_3$, sodium gluconate (*B*) or sodium cyclamate (*D*) instead of NaCl). *E*, comparison of the permeability ratios of I_{Cln} (○) and $I_{Cl,swell}$ (●). Data are presented as means ± S.E.M. from 6 oocytes for I_{Cln} and from 4 or 5 oocytes for $I_{Cl,swell}$. Cyclamate acted as a blocker of I_{Cln}, hence it is plotted at zero permeability. Gluc, gluconate; Cycl, cyclamate.

Table 1. $I_{Cln} \neq I_{Cl,swell}$

Chloride current	I_{Cln}	$I_{Cl,swell}$
Outward rectification	+++	+
Permeability	$NO_3^- > I^- > Cl^-$	$I^- \geqslant NO_3^- \geqslant Cl^-$
Effect of cyclamate	Block	Limited permeability
cAMP block	Voltage dependent	Voltage independent
Inactivation	pH independent	pH dependent
Activated by hypotonicity	No	Yes

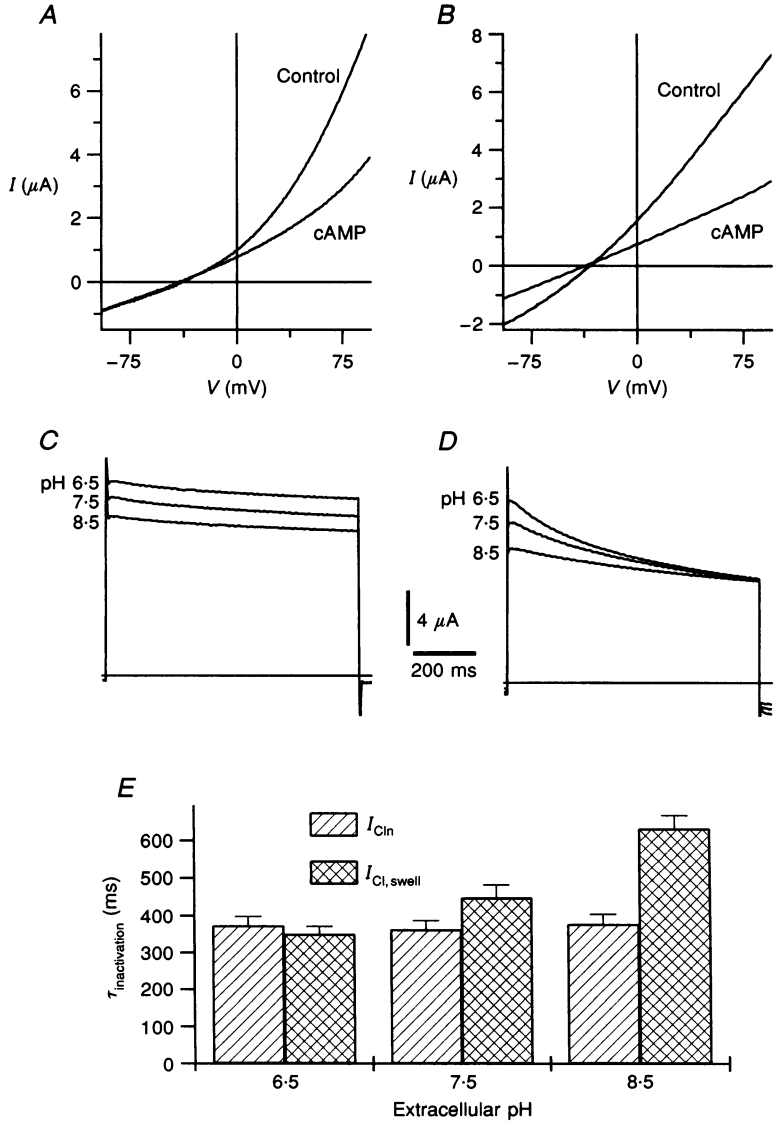

Figure 3. Additional differences between I_{Cln} and $I_{Cl,swell}$

A and *B*, effect of 5 mM extracellular cAMP on I_{Cln} (*A*) and $I_{Cl,swell}$ (*B*). Currents were measured with the ramp protocol. *C* and *D*, effect of extracellular pH on the inactivation properties of I_{Cln} (*C*) and $I_{Cl,swell}$ (*D*). Currents were measured with step protocol 2 in solutions adjusted to the indicated pH values. pH affected the inactivation kinetics of $I_{Cl,swell}$ but not of I_{Cln}. Higher pH values caused a decrease of the peak current amplitude for both currents. *E*, histogram showing the time constants of inactivation at +80 mV at different extracellular pH values for I_{Cln} ($n = 5$) and $I_{Cl,swell}$ ($n = 5$).

+80 mV ($n = 5$) *versus* only 3·1 ± 4·3% at −80 mV ($n = 5$). $I_{Cl,swell}$ is also sensitive to extracellular nucleotides, but in a voltage-independent way (Fig. 3*B*; Ackerman *et al.* 1994). cAMP (5 mM) blocked 53 ± 6% of the current at +80 mV ($n = 3$) *versus* 57 ± 9% at −80 mV ($n = 5$).

pH does not modulate the inactivation kinetics of I_{Cln}

A prominent feature of $I_{Cl,swell}$ is that the inactivation kinetics at positive potentials is modulated by the extracellular pH (Ackerman *et al.* 1994). Indeed, reducing the extracellular pH from 8·5 to 6·5 markedly accelerated the inactivation at +80 mV (Fig. 3*D* and *E*). Time constants of inactivation at +80 mV for $I_{Cl,swell}$ ($n = 5$) were (ms): 350 ± 20 (pH 6·5), 448 ± 34 (pH 7·5) and 632 ± 35 (pH 8·5). In contrast, extracellular pH did not affect the inactivation kinetics of I_{Cln} (Fig. 3*C* and *E*). Time constants of inactivation at +80 mV for I_{Cln} ($n = 5$) were (ms): 373 ± 25 (pH 6·5), 363 ± 24 (pH 7·5) and 378 ± 27 (pH 8·5). For I_{Cln} the only effect we noticed upon decreasing the extracellular pH was a small increase in the amplitude of the outward current (Fig. 3*C*; see also Gschwentner *et al.* 1994).

DISCUSSION

We have used *Xenopus* oocytes to study on the one hand I_{Cln}, the nucleotide-sensitive chloride current, and on the other $I_{Cl,swell}$, the swelling-activated chloride current. I_{Cln} was induced in collagenase-defolliculated *Xenopus* oocytes that had been injected with human pI_{Cln} RNA. $I_{Cl,swell}$ was activated by hypotonic stimulation of manually defolliculated or follicle-enclosed *Xenopus* oocytes. Although both currents shared some common properties such as outward rectification, slow inactivation at positive potentials, block by extracellular nucleotides and a $I^- > Cl^-$ permeability, we also noticed the following important differences (see also Table 1). (a) There is a significant difference in outward rectification with this parameter being more pronounced for I_{Cln}. (b) I_{Cln} has a clear preference for NO_3^- over Cl^-, whereas this is not the case for $I_{Cl,swell}$. (c) Cyclamate permeates to some extent through the channel carrying $I_{Cl,swell}$, whereas I_{Cln} is blocked by it. (d) The block by 5 mM extracellular cAMP is voltage independent for $I_{Cl,swell}$, whereas extracellular cAMP inhibits only the outward component of I_{Cln}. (e) Extracellular pH does not modulate the inactivation kinetics of I_{Cln}. In contrast, acidification of the extracellular medium accelerates the inactivation of $I_{Cl,swell}$ at positive potentials. (f) $I_{Cl,swell}$ is typically activated by reducing the tonicity of the extracellular medium. However, this procedure did not stimulate the amplitude of I_{Cln}. Furthermore, $I_{Cl,swell}$ cannot be activated in collagenase-defolliculated *Xenopus* oocytes, whereas I_{Cln} is readily expressed in enzymatically defolliculated oocytes.

In principle, one could argue that enzymatic defolliculation abolishes the volume sensor of *Xenopus* oocytes, which would explain why a hypotonic stimulus fails to trigger $I_{Cl,swell}$ or to modify the pI_{Cln}-induced current in defolliculated oocytes. However, this possibility seems unlikely as hypotonic stimuli have been reported to activate a K^+ conductance (Morin, Bond, Loo, Clarke & Bear, 1995) or a $^{86}Rb^+$ efflux pathway (Ratcliff & Ehrenfeld, 1994) in collagenase-defolliculated oocytes. We therefore favour the explanation proposed by Arellano & Miledi (1995) that follicular cells play an essential role in the activation of $I_{Cl,swell}$. In contrast, these cells are not required for successful expression of pI_{Cln}-induced currents.

Thus, I_{Cln} and $I_{Cl,swell}$ can be discriminated by biophysical (anion permeability sequence, degree of rectification and inactivation kinetics), pharmacological (voltage dependency of cAMP block) and biological (requirement of the follicular cell layer and of extracellular hypotonicity) criteria. These differences are, in our view, difficult to reconcile with $I_{Cl,swell}$ and I_{Cln} being identical currents in *Xenopus* oocytes. It is reasonable to extend this conclusion to other species as the *Xenopus* $I_{Cl,swell}$ very much resembles the volume-activated Cl^- current in mammals. Consequently, our observations do not support the hypothesis that pI_{Cln} is either the volume-activated channel or a direct cytosolic regulator thereof. They rather indicate that I_{Cln} and $I_{Cl,swell}$ are two dissociated phenomena that are not directly linked to each other. This conclusion is, at first sight, conflicting with previous observations that expression of pI_{Cln} is required for functional expression of $I_{Cl,swell}$ (Krapivinsky *et al.* 1994; Gschwentner *et al.* 1995). However, as we do not know the proper biological function of pI_{Cln}, we cannot *a priori* exclude the possibility that downregulation of pI_{Cln} interferes with general cellular processes such as biosynthesis of proteins, membrane sorting etc., which would indirectly affect the expression of $I_{Cl,swell}$. Also, the antisense oligonucleotide data were obtained on a mammalian cell line (Gschwentner *et al.* 1995), whereas our study deals with I_{Cln} and $I_{Cl,swell}$ in *Xenopus* oocytes.

We conclude that I_{Cln} and $I_{Cl,swell}$, although at first sight identical, are two different currents mediated by two distinct channels, the molecular nature of which remains to be elucidated. A critical review of the models that have linked pI_{Cln} directly to $I_{Cl,swell}$ is therefore required.

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