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Comparison of Amphibian and Human ClC-5: Similarity of Functional Properties and Inhibition by External pH

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Abstract: Loss of function mutations of the renal chloride channel, ClC-5, have been implicated in Dent's disease, a genetic disorder characterized by low weight proteinuria, hypercalciuria, nephrolithasis and, in some cases, eventual renal failure. Recently, our laboratory used an RT-PCR/RACE cloning strategy to isolate an amphibian cDNA from the renal epithelial cell line A6 that had high homology to human ClC-5. We now report a full-length native ClC-5 clone (xClC-5, containing 5′ and 3′ untranslated regions) isolated by screening a cDNA library from A6 cells that was successfully expressed in *Xenopus* oocytes. In addition, we compared the properties of xClC-5 and hClC-5 using isogenic constructs of xClC-5 and hClC-5 consisting of the open reading frame subcloned into an optimized Xenopus expression vector. Expression of the full-length "native" xClC-5 clone resulted in large, strongly rectifying, outward currents that were not significantly affected by the chloride channel blockers DIDS, DPC, and 9AC. The anion conductivity sequence was $NO_3^- > Cl^- = I^- > HCO_3^- >>$ glutamate for xClC-5 and $NO_3^- > Cl^- > HCO_3^- > I^- >>$ glutamate for hClC-5. Reduction of the extracellular pH (pH_o) from 7.5 to 5.7 inhibited outward ClC-5 currents by 27 ± 9% for xClC-5 and 39 ± 7% for hClC-5. The results indicate that amphibian and mammalian ClC-5 have highly similar functional properties. Unlike hClC-5 and most other ClC channels, expression of xClC-5 in oocytes does not require the removal of its untranslated 5′ and 3′ regions. Acidic solutions inhibited both amphibian and human ClC-5 currents, opposite to the stimulatory effects of low external pH on other ClC channels, suggesting a possibly distinct regulatory mechanism for ClC-5 channels.

Key words: Chloride channel — ClC-5 — Human — *Xenopus laevis* oocytes — Amphibian renal cell line — Outward rectification — pH

Introduction

ClC channels comprise a large family of voltage-gated chloride channels or putative chloride channels, which presently includes at least nine different members that are found in mammals (Jentsch, 1996; Thakker, 1997). ClC chloride channels have diverse roles including modulation of membrane excitability, and possibly functions in transepithelial ion transport and cell volume regulation. Mutations of three ClC channels are associated with inherited diseases in humans. For example, mutations of ClC-1, a major skeletal muscle chloride channel (Steinmeyer et al., 1991), lead to myotonia (Koch et al., 1992; Pusch et al., 1995*b*). Human ClC-Kb and ClC-5 mutations are associated with renal disorders, respectively, Bartter's syndrome type III (Simon et al., 1997) and Dent's disease (Lloyd et al., 1996, 1997*a*). Bartter's syndrome type III is characterized by hypokalemic alkalosis and hyperaldosteronism whereas Dent's disease is typified by kidney stone formation, hypercalciuria, and in some cases, eventually, renal failure. In functional expression studies of human ClC-5, Lloyd et al. (1997*b*) observed that mutations of this channel at sites previously identified in five unrelated families with Dent's disease led to an abolition or marked reduction in the expressed chloride current.

Despite the successful cloning of ClC-5 by several

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laboratories, a controversy has existed concerning its functional properties. Using CHO cells that were stably transfected with rat ClC-5 (rClC-5), Sakamoto et al. (1996) reported that expressed rClC-5 currents were more permeable to iodide than chloride and could be blocked by DIDS. Both Lloyd et al. (1996) and Steinmeyer et al. (1995) reported strong outward rectification of mammalian ClC-5 currents and a lack of sensitivity to DIDS and other chloride channel blockers. Moreover, Steinmeyer et al. (1996) also reported that rClC-5 had an anion conductivity sequence of $Cl^- > I^-$. Friedrich et al. (1999) recently reported identical properties for rat ClC-5 expressed in HEK 293 cells.

Recently, Lindenthal et al. (1997) used RT-PCR and RACE methods to isolate a cDNA clone from A6 cells that had high homology (70%) to human ClC-5 (hClC-5). Initial efforts to functionally express of this clone in *Xenopus* oocytes led to the induction of chloride currents that differed from those previously reported for human ClC-5 or rat ClC-5 (Steinmeyer et al., 1995; Lloyd et al., 1996). Specifically, this current was blocked by DIDS and was more permeable to iodide than chloride. Subsequent studies (Schmieder et al., 1998; Mo et al., 1998) revealed that this current was due to an endogenous current similar to that found by Buyse et al. (1997). The latter investigators reported that ClC-6 and pI_{CLn} expression in oocytes led to the activation of endogenous currents that were sensitive to chloride channel blockers and were more permeable for iodide than chloride.

The purpose of the present study was twofold. First, the full-length native xClC-5 cDNA sequence (including the 5′ and 3′ untranslated regions) was isolated by screening a cDNA library from *Xenopus* cultured renal epithelial (A6) cells and expressed in *Xenopus* oocytes. Second, we directly compared the electrophysiological properties of amphibian and mammalian ClC-5 by expressing isogenic constructs of xClC-5 and hClC-5 in paired batches of oocytes. The constructs consisted of the open reading frame for each protein in an expression vector that was optimized for the *Xenopus* oocyte expression system. The voltage dependence and sensitivity of the currents to various channel blockers and anions, including bicarbonate, were compared. In addition, since several members of ClC family are stimulated by extracellular pH (pH_o) including ClC-1 (Rychkov et al., 1996, 1997; Astill et al., 1996) and ClC-2 (Jordt & Jentsch, 1997; Pusch & Jentsch, 1994), we also compared the effects of solution pH on xClC-5 and hClC-5 conductances. Specifically, we asked the following questions: (i) What are current-voltage relationships and gating properties of functionally expressed amphibian and human ClC-5? (ii) Are ClC− channels permeable to bicarbonate? What are the relative anion conductances and sensitivities to chloride channel blockers? and (iii) Does external pH modulate xClC-5 and hClC-5 currents similarly? Preliminary findings of part of these investigations were published previously in abstract form (Mo et al., 1998).

Materials and Methods

mRNA Isolation, cDNA Library Construction, Screening, and Sequencing

To construct a cDNA library for A6 cells, total RNA (from A6 cells grown on plastic Petri dishes) was isolated using the method of Chirgwin et al. (1979). Poly(A^+)RNA was prepared using a commercially available kit (PolyA$^+$Quick; Stratagene). A cDNA library was then constructed in the ZAP-Express Lambda vector (Stratagene) according to the manufacturer's protocols. Briefly, double-stranded cDNA was prepared and cDNA > 600 bps was directionally cloned into the *Eco RI/Xho I* site of the Zap-Express vector. The resultant library of 1×10^6 recombinants was amplified, and subsequently, 1×10^6 phage were plated and screened using probes for ClC channels generated from previous RT-PCR experiments. Hybridizing clones were plaque-purified using three rounds of selection. Plaque-pure clones were subjected to in vivo excision and the resulting cDNA clones were analyzed by restriction analysis and sequenced in both directions using an automated DNA sequencer (Applied Biosystems model 373) and synthetic primers (Recombinant DNA laboratory, University of Texas Medical Branch). The construct for this clone will be referred to as "native xClC-5" and unless otherwise noted, contains the 5′ and 3′ untranslated regions in the PBK-CMV phagemid vector.

cDNA clones and cRNA Preparation

For comparison of human and amphibian ClC-5 properties, similar "isogenic" constructs were prepared. cDNA encoding the open reading frame of human ClC-5 cDNA in the pTLN oocyte expression vector (Lloyd et al., 1995; Jentsch et al., 1996) was generously provided by Dr. Thomas Jentsch. The pTLN vector is a modified version of the pSP64T expression vector containing *Xenopus* globin untranslated regions (Krieg & Melton, 1984). For xClC-5, the same expression vector was used with the following restriction sites substituted for the *Nco I − Bgl II* region of the polylinker: 5′ *Pvu II, Sph I, Pst I, Sal I, Xba I, BamH I, Sma I, Kpn I, Sst I, EcoR I, Cla I, EcoR V, Bgl II.* To generate the xClC-5 cDNA construct, an *EcoR I/ BamH* I fragment containing a Kozak sequence and the entire coding region of xClC-5 was cloned into the *EcoR I*/*Bgl II* sites the above expression vector. These constructs will be referred to as hClC-5-ORF and xClC-5-ORF, for the human and *Xenopus* ClC-5 clones, respectively.

For in vitro transcription, ~1 μg of oocyte expression vector containing *Xenopus* ClC-5 (xClC-5-ORF) or human ClC-5 (hCLC-5-ORF) cDNA in the pOocyte vector was linearized with *Mlu I* and the native xClC-5 in the PBK-CMV vector was linearized with *Not I* (New England BioLabs, Beverly, MA). The linearized plasmids were extracted with phenol-chloroform then transcribed using a commercially available kit (mMessage mMACHINE™ SP6 or T3 Kit; Ambion, Austin, Texas) to produce capped cRNA. The yield from each transcription (~10 μg) was dissolved in diethyl pyrocarbonate-treated water and stored at −80°C.

Functional Expression in *Xenopus* Oocytes

Oocyte Preparation and Injection

For expression studies, oocytes were removed and defolliculated by collagenase digestion (Crescent Chemical, Hauppaug, NY; for details,

see Lindenthal et al., 1997). Three to four hours after defolliculation, Stage V/VI oocytes were injected with either 50 nl of nuclease-free water, or 50 nl of a 0.1–0.2 ng/nl cRNA solution (total cRNA per injection = 5–10 ng) as prepared above. Oocytes were incubated in modified Barth's solution (in mM: 88 NaCl, 1.0 KCl, 2.4 $NaHCO_3$, 5 Tris/HCl, 0.82 $MgSO_4$, 0.33 $Ca(NO_3)_2$, 0.41 $CaCl_2$, pH 7.5). After 2–3 days at 17°C, they were mounted in a recording chamber and impaled with current and voltage measuring microelectrodes. The membrane potentials were allowed to become stable before recording, usually for a period of approximately 5 minutes.

Electrical Recordings

Microelectrodes consisted of borosilicate glass (Garner Glass, Claremont, CA) pulled to tip diameters of 1–5 μm (using a model P-87 puller; Sutter Instruments, Palo Alto, CA). The electrodes were backfilled with 3 M KCl solution and had tip resistances of 0.5–2 MΩ. The bath ground and reference electrodes consisted of 3 M KCl agar bridges connected to Ag-AgCl wires. All electrodes were led to a high compliance automatic voltage clamp (OC-725B amplifier; Warner Instrument) interfaced to an A/D converter (TL-1; Axon Instruments, Foster City, CA) and laboratory microcomputer (Dell 486). Potential measurements were corrected for solution junction potentials.

Data acquisition and analysis were performed using commercially available software (Clampex, pClamp6; Axon Instruments, Foster City, CA). Oocytes were clamped at a −40 mV holding potential and 350 msec voltage steps from −140 to +100 mV in 20 mV increments were applied. All recordings were performed at room temperature.

Solutions

Solutions consisted of ND96 buffer containing (in mM): 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, and 5 HEPES titrated with NaOH to pH 7.5. In ion replacement experiments, chloride was partially replaced (80 mM) by equal concentrations of iodide, glutamate, nitrate, or bicarbonate. Sodium was similarly replaced by potassium or Tris. To investigate the effect of pH over the range of 5.3–9.5, the solution was buffered with MES or Tris and titrated to the desired pH value by HCl or NaOH addition. Solutions containing bicarbonate (80 mM) were maintained at pH 7.4 by bubbling with a mixture of 19% CO_2 and 81% air.

Methods for Calculation of Relative Slope Conductances and Statistics

Unless otherwise noted, steady-state currents were used to calculate current-voltage relationships (*I–V* curves). The inward conductance was calculated as the slope of current values between −140 and −80 mV and outward conductance was determined as the slope between +80 to +100 mV potentials. Data are presented as means ± SEM unless otherwise indicated. Statistical analyses were done by paired *t*-tests as appropriate.

The outward rectification of the ClC-5 channels precluded calculation of the relative anion permeability from reversal potential measurements. For this reason, the relative slope conductances in various anion solutions were compared by normalizing the slope conductances to the control value in ND96 solution.

Results

As a first step in these investigations, xClC-5 properties were determined using cRNA made from the native xClC-5 clone. These results were then compared to those obtained for the isogenic constructs, xClC-5-ORF and hClC-5-ORF. Lastly, the ion dependence, channel blocker and pH effects on these currents were assessed.

Isolation and Characterization of "Native" xClC-5 from A6 cDNA Library

To determine the sequence of native xClC-5, an amplified A6 cDNA library was screened with a 2 kb probe based on the xClC-5 sequence originally obtained by RT-PCR/RACE by Lindenthal et al. (1997). Ten positive clones were isolated and one full-length cDNA of 2901 bp was obtained and sequenced. As in the earlier clone, the open reading frame consisted of 2424 bp, predicting an 808 amino acid translation product with a molecular mass of approximately 90 kDa (*see* Fig. 1 for a comparison of xClC-5 and hClC-5 predicted amino acid sequences). The corrected sequence has been submitted to Genebank (accession number AF063904).

Briefly, within the coding region, there were three differences in the nucleotide sequence: (i) a thymindine at position 1177 instead of guanosine (resulting in a leucine at amino acid position 393 instead of valine), (ii) a thymindine at position 1768 instead of adenosine (resulting in a leucine at amino acid position 590 instead of an isoleucine) and (iii) an adenosine instead of guanosine at 1907 (resulting in a glutamine instead of a glycine at amino acid position 636). Presumably these errors were due to RT-PCR artifacts. Two errors were also detected in the previously reported predicted amino acid sequence. A tyrosine is present at position 294 instead of a threonine and an alanine was encoded at position 602 instead of a serine. Consequently, the amino acids at these five positions now match those in the corresponding sequence for human ClC-5 (Fisher et al., 1995).

The untranslated regions of the native xClC-5 cDNA sequence also differed from the previously reported sequence. The 5′ untranslated region consisted of 320 nucleotides and the 3′ untranslated region consisted of 159 nucleotides. The most notable difference in the 5′ UTR sequence of the native clone was the absence of a region of 33 nucleotides that was incorporated immediately before the ATG site in the previous RT-PCR/RACE generated clone. We assume this difference reflects an error in the RACE generated sequence for the 5′ UTR end. In contrast, the 3′ UTR of the clones differed in only a few nucleotides.

Functional Expression of Native xClC-5

To characterize the conductance properties of "native xClC-5" (i.e., the above cDNA clone isolated from the A6 cDNA library and containing the 5′ and 3′ untranslated regions) this construct was linearized, transcribed as described above, and the resulting cRNA was injected into *Xenopus* oocytes (for further description, *see* Mate-

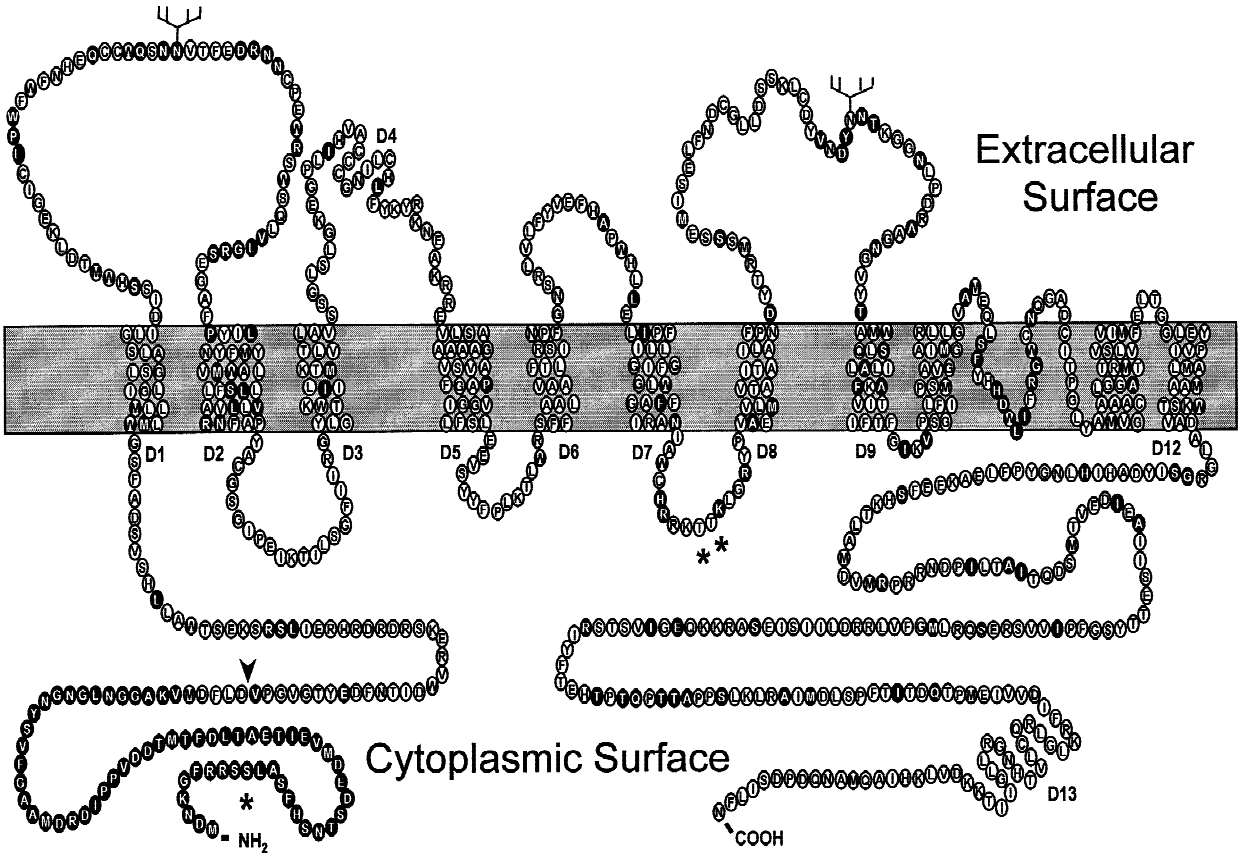

Fig. 1. Comparison of predicted amino acid sequences of native xClC-5 isolated from A6 cDNA library and human ClC-5. For convenience in comparing to hClC-5, the sequence has been presented according to the hypothetical model of Lloyd et al. (1996). Black circles indicate amino acids that differ from hClC-5 and white circles indicate identical amino acids. Potential N-linked glycosylation sites are indicated by branched motifs; potential phosphorylation sites are marked by asterisks.

rials and Methods). As summarized in Fig. 2*A*, within two days, a distinct outwardly rectifying current was observed in ~60% of the injected oocytes (2.9 ± 0.5 μA; $n = 7$ from a total of 12 oocytes, 3 frogs).

Comparison of the Expression of Human and Amphibian ClC-5

It has long been recognized that some ion channels show improved expression when subcloned into certain expression vectors (*cf.*, Swanson & Folander, 1992). For example, expression of the rat cardiac Na^+ channel required using cRNA transcripts from cDNA subcloned into the pSP64T expression vector (Cribbs et al., 1990) which is optimized for the *Xenopus* oocyte expression system (*see* Materials and Methods). For this reason, we next compared xClC-5 to human ClC-5 using a similar expression vector containing the open reading frame for

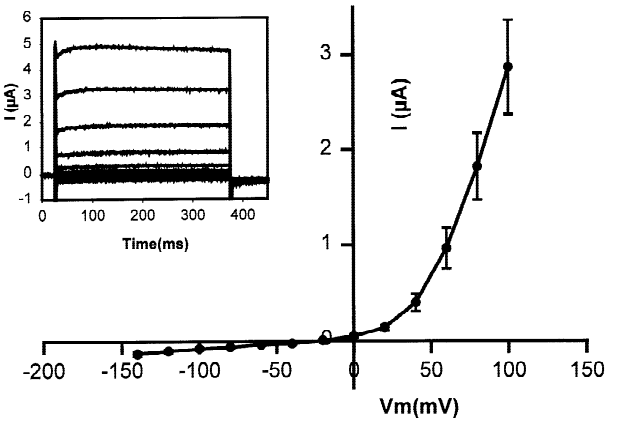

Fig. 2. Functional expression of native xClC-5 in *Xenopus* oocytes ($n = 7$). The smooth curve indicates the mean current-voltage relationship. *Insert:* Data from a typical experiment showing the time course of current responses to 350 msec voltage steps from −140 to +100 mV.

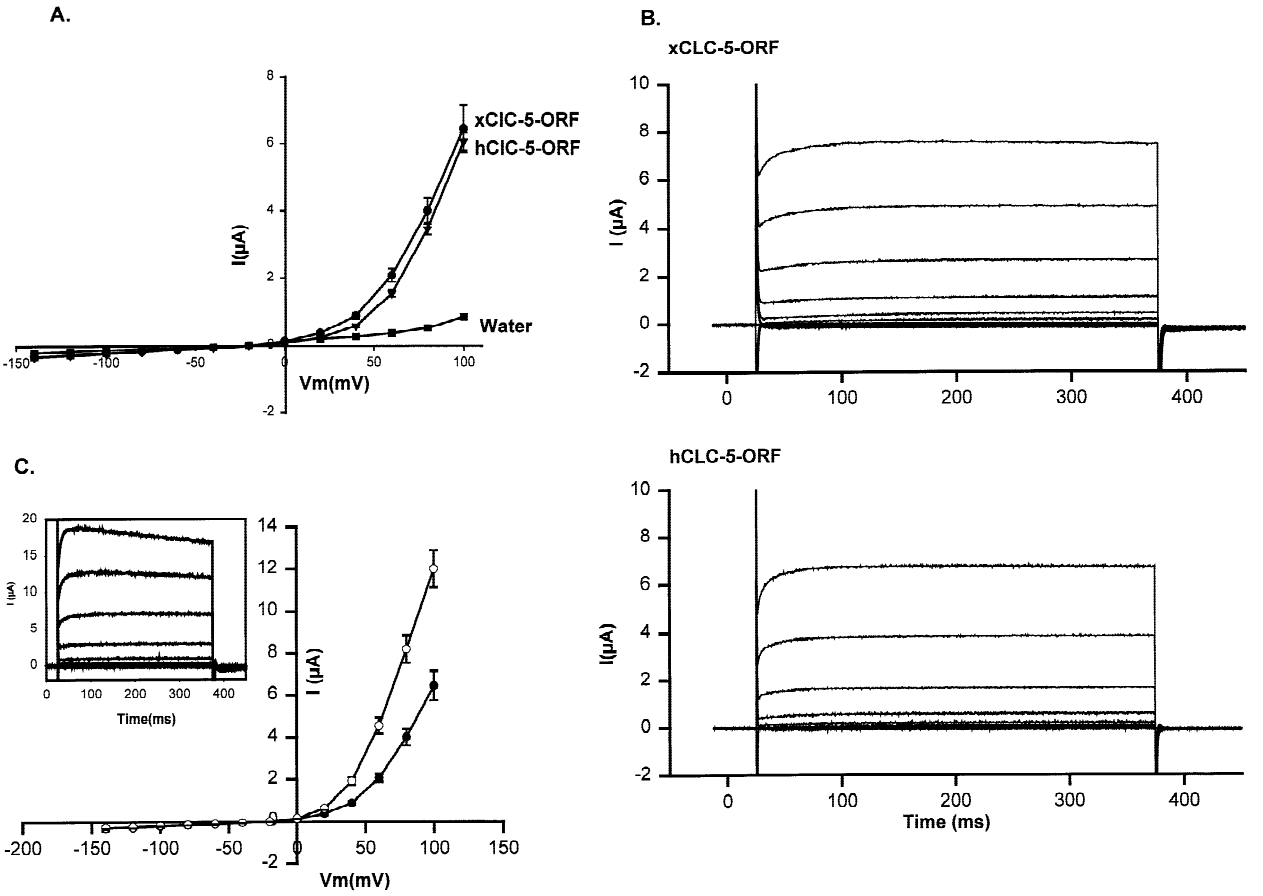

Fig. 3. Comparison of functional expression of amphibian and mammalian ClC-5 in *Xenopus* oocytes. (*A*) Steady-state *I–V* relationship for oocytes injected with amphibian ClC-5 RNA (xClC-5-ORF; circles, $n = 19$), human ClC-5 RNA (hClC-5-ORF; triangles, $n = 38$) or water (squares, $n = 30$). Similar data were obtained for 7 paired measurements of xClC-5-ORF and hClC-5-ORF in 6 batches of oocytes from the same frogs. (*B*) Typical time traces for xClC-5-ORF (upper panel) and hClC-5-ORF (lower panel) currents in oocytes from the same frog. (*C*) Mean *I–V* relationship and a typical time trace (insert) illustrating time-dependent decreases in xClC-5-ORF currents ($n = 8$; open circles) compared to the mean *I–V* of time-independent xClC-5-ORF currents (filled circles; data from Fig. 3*A*). For further details concerning cDNA constructs and expression vectors, see text.

these proteins as described (*see* xClC-5 -ORF and hClC-5-ORF above).

Although some results have been pooled for the purpose of illustration, the results of all experiments comparing hClC-5-ORF and xClC-5-ORF expression were repeated and confirmed on paired batches of oocytes from the same frogs. In experiments using cRNA made from the xClC-5-ORF cDNA construct, a large outwardly rectifying current was observed in 87% of the oocytes ($n = 27$, total number of oocytes $= 31$, 5 frogs) two days following injection. The current-voltage relationship was essentially identical to that obtained with the native xClC-5 clone. Notably, amounts of cRNA as low as 2 ng led to expression within 24 hr and currents as large as 40 μA were observed after three days following injection with 5 ng of cRNA (*see* example in Fig. 3*A* and *B*).

Similar to xClC-5-ORF, large, highly reproducible, currents were obtained for 78% of oocytes injected with cRNA for hClC-5-ORF ($n = 38$, total number of oocytes $= 49$, 8 frogs). Figure 3*A* illustrates the average current-voltage relationships for xClC-5 and hClC-5 injected oocytes compared to water-injected oocytes. Similar data were obtained for 7 paired measurements of these constructs expressed in 6 batches of oocytes from the same frogs. The mean maximum currents (at 100 mV) were 6.5 ± 0.7 μA for xClC-5-ORF ($n = 19$) and 6.1 ± 0.3 μA for hClC-5-ORF ($n = 38$) compared to 0.8 ± 0.1 μA for water-injected oocytes ($n = 30$). On average, the expressed ClC-5 currents were 8–10 times larger than current for water-injected oocytes.

As in the case of the current for the native xClC-5 clone (recall Fig. 2*A*), both currents showed strong outward rectification and were approximately 30–40 times

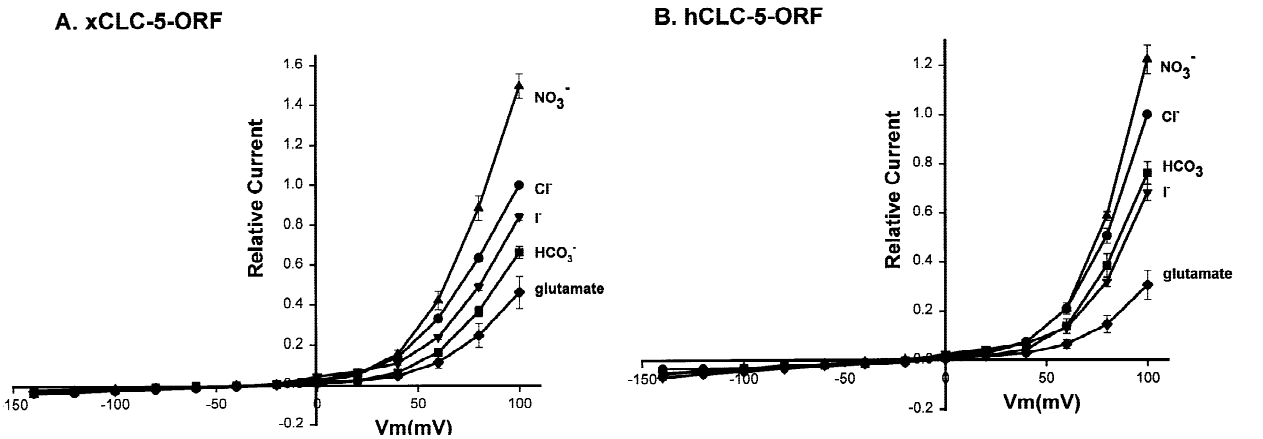

Fig. 4. Relative *I–V* relationship for expressed ClC-5 clones in the presence of different anions. Panels *A* and *B* show the relative current voltage relationships for xClC-5 and hClC-5, respectively. The current values from individual experiments were normalized to control current at +100 mV. Chloride (circles) was partially replaced (80 mM) by nitrate (triangles), iodide (inverted triangles), bicarbonate (squares), or glutamate (diamonds), respectively.

larger at +100 mV voltage than at −100 mV. The time-courses of the xClC-5-ORF and hClC-5-ORF currents were similar (Fig. 3*B*) and did not show significant time-dependent decreases. These time-courses data are in excellent agreement with previous studies of hClC-5 (Steinmeyer et al., 1995).

As shown in Fig. 3*C* (*see* insert), some xClC-5-ORF currents (8 out of 27 oocytes) showed a slightly time-dependent inactivation. However, these currents were significantly larger (12.0 ± 0.9 μA at +100 mV; $P < 0.05$) than the hClC-5-ORF or xClC-5-ORF currents described above. It is possible that this difference may reflect the activation of an additional endogenous current, although we did not evaluate this factor.

Ion Dependence of the ClC-5 Induced Currents

The resting membrane potential (V_m) averaged −38 ± 2 mV ($n = 30$) for water-injected oocytes, but was depolarized to −25 ± 2 mV ($n = 27$) and −26 ± 2 mV ($n = 37$), for oocytes injected with cRNA for xClC-5-ORF or hClC-5-ORF, respectively. These values are nearly equal to the chloride equilibrium potential (~−30 mV), assuming an intracellular activity of 33 mM (Barish, 1983). This result suggests that the chloride selectivity of the oocyte membrane was increased following expression of xClC-5 or hClC-5, or alternatively, the depolarization might reflect the induction of a poorly selective cation channel.

To test for cation permeability, we partially replaced (80 mM) the bathing solution sodium by potassium or Tris. Replacement of sodium by potassium or Tris did not significantly affect the outward current or the relative slope conductances for both xClC-5-ORF or hClC-5-ORF (*data not shown*). These experiments indicate that a cation current is not responsible for the ClC-5 induced currents.

As shown in Fig. 4*A* and *B*, the xClC-5-ORF and hClC-5-ORF currents were highly permeable to chloride. Replacement of 80 mM chloride by glutamate reduced the outward current and outward slope conductance (i.e., decreased the chloride influx) for both currents. The outward current at +100 mV decreased by 54 ± 8% ($n = 8$) for xClC-5 and by 69 ± 6% ($n = 10$) for hClC-5. In addition, the outward slope conductance was significantly reduced by 40 ± 4% ($n = 8$) and 63 ± 7% ($n = 10$) for xClC-5-ORF and hClC-5-ORF, respectively. Restoration of chloride to the bathing solution returned the currents to their initial values (*data not shown*). These results, together with the lack of cation permeability above, are consistent with a chloride-permeable conductive pathway. In agreement with Steinmeyer et al. (1995), deactivation of the currents was essentially instantaneous. Consequently, it was not possible to measure a shift in the reversal potentials for xClC-5 or hClC-5 from tail currents under these conditions. Reduction of the chloride concentrations to values as low as 2 mM had no significant effect on the gating of ClC-5 currents (*data not shown*). These findings are unlike those of Pusch et al. (1995) who demonstrated that gating of ClC-0 channels is dependent on the extracellular chloride concentration.

Previous studies have shown that endogenous currents and expressed hClC-5 chloride currents can be distinguished by their anion conductivity sequence (Steinmeyer et al., 1995). Table 1 summarizes relative outward-slope conductance data for the three ClC-5 constructs. For all three clones, the relative anion slope conductance values compared to chloride were significantly higher for nitrate and lower for bicarbonate. In-

Table 1. Relative slope conductances for xClC-5 or hClC-5 in different anion solutions (G_x/G_{Cl})$^{\alpha}$

	"Native" xClC-5	xClC-5-ORF	hClC-5-ORF
Nitrate	1.64 ± 0.07*	1.62 ± 0.08*	1.43 ± 0.08*
Iodide	0.95 ± 0.04	0.97 ± 0.03	0.74 ± 0.04$^{\beta,*}$
Bicarbonate	0.87 ± 0.02*	0.83 ± 0.03*	0.83 ± 0.06*

$^{\alpha}$ G is defined as slope conductance between +80 to +100 mV
$^{\beta}$ $P < 0.05$ compared to xClC-5 (ORF);
* $P < 0.05$ compared to ND96 chloride solution.

terestingly, the relative slope conductance for iodide, which was significantly decreased in hClC-5 clones, was not significantly reduced in both xClC-5 clones. Despite this difference, the ClC-5 induced currents have a $Cl^- \geq I^-$ slope conductance sequence. This feature is illustrated in Fig. 4*A* and *B*, which shows that iodide substitutions had less effect on xClC-5-ORF currents than hClC-5-ORF currents at high potential values whereas glutamate strongly blocked both currents. These findings are unlike those for endogenous chloride currents or for currents induced by expression of xClC-5 constructs subcloned in the pGEM-5Zf(+) expression vector, which have a selectivity sequence of $I^- > Cl^-$ (*data not shown*, *see* Lindenthal et al., 1997).

Blockers

To assess the pharmacological sensitivity of the expressed ClC-5 currents, several chloride channel blockers were tested including DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid), 9-AC (anthracene-9-carboxylic acid), and DPC (diphenylamine-2-carboxylic acid). Despite the use of several different incubation periods and pulse protocols, no effects of DIDS (1 mM), 9-AC (2 mM), and DPC (1 mM), on the ClC-5 currents were detected in agreement with previous results for rClC-5 (Steinmeyer et al., 1995; *data not shown*).

Effects of pH

Several studies have reported that pH is a potent regulator of ClC channels. Jordt and Jentsch (1997) recently demonstrated that decreases in extracellular pH (pH_o) activate the inward-rectifying ClC-2 channel, which is also transiently activated followed by inhibition during extreme external acidification (pH = 3.0; Furukawa et al., 1998). In addition, the gating properties of ClC-1 are also affected by pH_o (Rychkov et al., 1996). For this reason, we tested the effects of pH_o on expressed xClC-5 or hClC-5 currents. Reduction of pH_o from 7.5 to 5.7 decreased both currents (27 ± 9%; $n = 5$ and 39 ± 7%; $n = 6$, for xClC-5-ORF and hClC-5-ORF, respectively at +100 mV). Currents decreased within 1 min following exposure to acidic solutions and remained inhibited for at least 15 min (the maximum time period measured). The membrane potential significantly depolarized from −27 ± 5 mV to −3 ± 3 mV for xClC-5 ($P < 0.01$, $n = 5$) and from −28 ± 4 mV to −16 ± 2 mV for hClC-5 ($P < 0.05$, $n = 6$). These effects were fully reversible upon return to normal bathing solutions with pH = 7.5. In contrast, membrane potentials in water-injected oocytes were not significantly changed by acidic solutions and the current responses were transient or variable (*data not shown*). Preliminary measurements of intracellular pH (pH_i) using ion-sensitive microelectrodes in xClC-5 or water injected oocytes indicate that pH_i was not significantly affected by exposure to acidic solutions ($\Delta pH_i = -0.03 \pm 0.10$; $n = 4$; P. Fong, G. Cooper & N.K. Wills, *unpublished observations*).

Figure 5*A* and *B* show the *I–V* relationships for the expressed xClC-5-ORF and hClC-5-ORF currents for $pH_o = 5.3$, 7.5 and 8.7. The slope conductances were calculated as described in Material and Methods and were normalized to the value at $pH_o = 7.5$. As indicated in Table 2, reduction of the extracellular pH from 7.5 to 5.3 significantly decreased the relative slope conductances of the outward current (to 79 ± 8% and 72 ± 5% for xClC-5-ORF and hClC-5-ORF, respectively; $n = 6$; $P < 0.05$). In contrast, the slope conductances of the inward current for both ClC-5 currents were not significantly changed. Alkaline pH_o also had no significant effects on the outward or inward xClC-5-ORF currents and slope conductances, although the current values for hClC-5-ORF were slightly increased at +100 mV (16 ± 3%, $n = 6$, $P < 0.05$). The time-dependence of the currents was not affected by acidic pH_o (*data not shown*).

Figure 6 summarizes the effects of bathing solution pH on the relative current of xClC-5-ORF and hClC-5-ORF. Current at +100 mV for each experiment was normalized to the corresponding value at pH 8.7. The sigmoidal line illustrates the results of a fit of the data using a least-squares curve fitting algorithm and the Hill equation as follows:

$$I = I_{min} + (I_{max} - I_{min})/(1 + ([H^+]/K_d)^n) \quad (1)$$

where I is the normalized currents to the current at pH 8.7, I_{max} and I_{min} are the maximum and minimum relative current, respectively, $[H^+]$ is the proton concentration (which is equal to 10^{-pH}), K_D is the dissociation constant (which is equal to 10^{-pK_D} or $-\log K_D = pK_D$), and n is the Hill coefficient (Kajita et al., 1997).

The mean dissociation constants K_d and Hill coefficient n were estimated from fits of individual experiments and did not significantly differ for xClC-5 and hClC-5. The mean pK_D for individual experiments was pH 6.2 ± 0.1 for xClC-5 ($n = 6$) and 6.4 ± 0.1 for hClC-5 ($n = 6$). The corresponding Hill coefficients were 1.2 ± 0.3 for xClC-5 and 1.0 ± 0.1 for hClC-5.

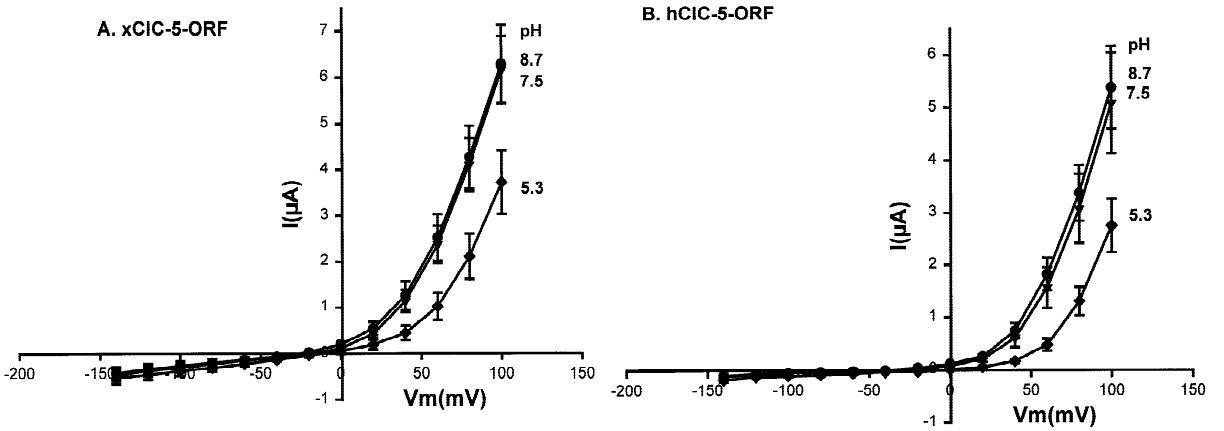

Fig. 5. Comparison of the effects of decreasing or increasing pH_o on the *I–V* relationship of xClC-5-ORF (*A*, $n = 6$) and hClC-5-ORF (*B*, $n = 6$) currents. Control data at pH 7.5 were obtained immediately prior to the solution change and are indicated by triangles. Paired measurements were obtained 1 min following randomized changes of the bath pH to 8.7 (circles), and 5.3 (diamonds).

Table 2. Effects of pH on relative inward and outward relative slope conductances*

pH	9.5	8.7	5.3
xClC-5-ORF			
Outward	100 ± 4	99 ± 3	79 ± 8[a]
Inward	86 ± 14	90 ± 7	90 ± 15
$N = 6$			
hClC-5-ORF			
Outward	105 ± 5	101 ± 4	72 ± 5[b]
Inward	89 ± 7	100 ± 27	142 ± 23
$N = 6$			

* slope conductances are normalized to the values at $pH_o = 7.5$.
[a] $P < 0.05$ compared to pH 7.5 for xClC-5 outward conductance; paired *t*-test.
[b] $P < 0.05$ compared to pH 7.5 for hClC-5 outward conductance; paired *t*-test.

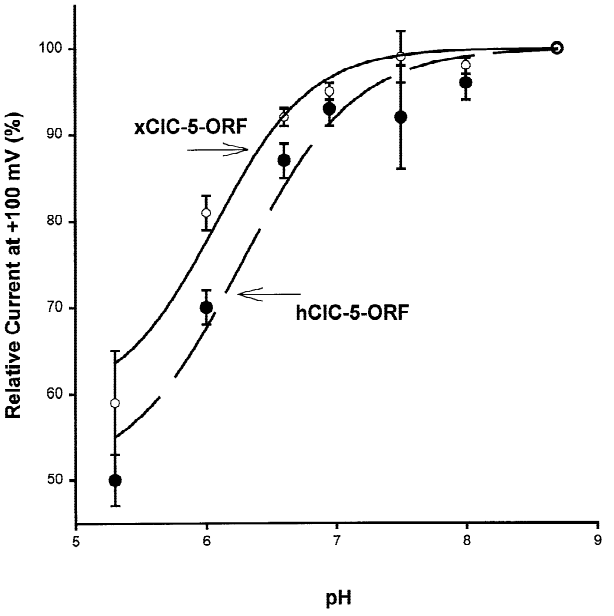

Fig. 6. Effects of pH_o on the relative outward currents. The individual currents at voltages +100 mV were normalized to values at $pH_o = 8.7$ for the same cell. Open circles indicate data for xClC-5 and filled circles are hClC-5. The sigmoidal curves represent the results of fits of the mean relative currents by a least-squares regression analysis to the Hill equation (Eq. 1) for xClC-5 (solid line) or hClC-5 (dashed line), respectively. The pK_D value for mean relative currents was 6.1 for xClC-5-ORF and 6.3 for hClC-5-ORF. The corresponding Hill coefficients were 1.2 for xClC-5-ORF and 1.0 for hClC-5-ORF (for results of fits of individual experiment, *see text*). Note that both xClC-5-ORF and hClC-5-ORF currents decreased at pH values below 7.5.

Discussion

The importance of the present findings is fourfold. First, the results represent the first cloning of a "native" ClC channel from an A6 cDNA library. The significance of this result is that native xClC-5, had properties similar to hClC-5 and unlike nearly all other ClC channels, can be expressed without removal or replacement of its untranslated regions. Second, the present data are the first direct comparison of the properties of human ClC-5 (Lloyd et al., 1996) and amphibian ClC-5 currents. More importantly they are the first demonstration of a bicarbonate permeability of these channels. Lastly, a novel inhibitory regulation of ClC-5 channels by external H^+ was described that is opposite to the effects of acidic bathing solutions on other ClC channels.

Confirmation of ClC-5 Properties

The present results for ClC-5 expression in oocytes extend and confirm previous results reported for oocyte

expression of hClC-5 (Lloyd et al., 1995), rClC-5 (Steinmeyer et al., 1995), and xClC-5 (Schmieder et al., 1998). However, they conflict with previous studies of rClC-5 stably expressed in CHO cells (Sakamoto et al., 1996). The latter investigators reported that rClC-5 was blocked by DIDS and that was more permeable for iodide than for chloride.

In a previous study (Lindenthal et al., 1997), we reported that xClC-5 constructs containing 5′ and 3′ UTRs obtained by RT-PCR/RACE and subcloned in the pGEM-5Zf(+) expression vector also induced a current that was more permeable to iodide than chloride and was blocked by DIDS. These properties were similar to those of an endogenous current subsequently described by Buyse et al. (1997). Interestingly, oocytes injected with cRNA transcribed from xClC-5 cDNA in the pGEM-5Zf(+) vector displayed this endogenous current more frequently following incubation at elevated temperatures (one hr in a 28°C). Buyse et al. (1997) observed similar endogenous currents in oocytes injected with cRNA for ClC-6 after a similar incubation protocol. The reasons for the activation of this endogenous current are unclear.

Using the xClC-5-ORF cDNA contained in the pGEM-5Zf(+) vector, Schmieder et al. (1998) recently reported that xClC-5 in vitro translation and expression levels in oocytes (as detected by Western blots) were decreased compared to constructs using an expression vector similar to that used in the present study (in which the ORF is flanked by the 5′ and 3′ UTRs of the *Xenopus* beta globin gene). ClC-5 protein translation levels were not reported by Sakamoto et al. (1996). Therefore, it is unclear whether similar problems can explain their results.

Comparison of xClC-5 and hClC-5 Properties

Confirmation of ClC-5 Outward Rectification and Blocker Insensitivity

Expression of cRNA made from either of the isogenic xClC-5-ORF or hClC-5-ORF constructs in oocytes led to a depolarization of the resting membrane potential towards the chloride equilibrium potential and was accompanied by large, outwardly rectifying currents. Both currents had similar current voltage-relationships and showed little time-dependence. These results are essentially identical to those of Lloyd et al. (1996) for hClC-5-ORF and confirm and extend those of Schmieder et al. (1998) for xClC-5 expressed in oocytes using a similar expression vector. Further experiments are needed at the single channel level to determine whether channel rectification results from channel gating or from saturation of the single channel conductance.

Unlike most other ClC channels (Fong & Jentsch, 1994), both amphibian and human ClC-5 currents were insensitive to chloride channel blockers, including DIDS, 9-AC, and DPC. These properties also agree with rClC-5 expressed in oocytes (Steinmeyer et al., 1995).

Similarity and Differences in Human and Amphibian ClC-5 Expression

Anion conductance. Both ClC-5 conductances were selective for anions rather than cations. For hClC-5, the anion slope conductance sequence $NO_3^- > Cl^- > HCO_3^- > I^- >>$ glutamate was similar to the sequence reported for rat ClC-5 expressed in oocytes, ($NO_3^- > Cl^- > I^- >>$ glutamate; Steinmeyer et al., 1995). A new finding of the present study was a conductance in bicarbonate solutions that was relatively low compared to chlorine for both xClC-5 and hClC-5. The effects of bicarbonate were rapid and complete within less than 1 min following the solution change. In contrast, preliminary pH-sensitive microelectrode experiments of xClC-5 (native clone) or water-injected oocytes (P. Fong, G. Cooper and N.K. Wills, *unpublished*) indicated that the intracellular pH decreased ($\Delta pH_i = 0.65 \pm 0.09$) over a period of four minutes after replacement of chloride (80 mM) by bicarbonate. Further studies are needed to determine the possible effects if intracellular pH on ClC-5 currents.

Although both hClC-5-ORF and xClC-5-ORF both show larger relative currents in chloride compared to iodide solutions, the relative slope conductance for xClC-5-ORF was similar in iodide solution and chloride solution (i.e., $G_{Cl} = G_I$). Aside from this difference, the conductance sequence for xClC-5-ORF ($NO_3^- > Cl^- = I^- > HCO_3^- >>$ glutamate) was similar to that of hClC-5-ORF. The reason for this apparent anion dependence of the rectification is presently unclear. In preliminary experiments, we could find no evidence for a possible inhibitory effect of iodide on its own permeation as reported previously for the CFTR chloride channel (Tabcharani et al., 1997).

Recently Duan et al. (1997) demonstrated that mutation of a single amino acid at the end of the transmembrane domains of ClC-3 (N579K) resulted in an alteration in the anion selectivity of this channel. The homologous amino acids in the xClC-5 and hClC-5 sequences are identical at this site. Thus any differences between xClC-5-ORF and hClC-5-ORF are not due to this residue. Experiments are presently underway to determine whether mutations of this conserved region of the amino acid sequence of xClC-5 have an effect on its anion conductivity.

In most experiments, the amplitude and time-course of the xClC-5-ORF currents expressed in oocytes were essentially identical to that of hClC-5-ORF. However, in

some experiments ($n = 8$ out of 27), the xClC-5 outward currents were nearly twice as large (mean value at +100 mV = 12 ± 1 μA compared to 6 ± 1 μA) and a slight (7%) decrease was noted at large positive potentials. This decrease was not due to poor voltage clamp since our voltage clamp had high compliance (i.e., capable of passing ±100 μA current; *see* specifications for the Model OC-725B oocyte clamp by Warner Instrument). We also note that comparable cRNA concentrations were injected for all experiments, the oocytes were incubated identically, and the same batches of oocytes were used for both the xClC-5 and hClC-5 experiments. Several factors could account for such a result, including activation of an additional endogenous anion or cation current or changes in the transmembrane chloride concentration gradient.

Comparison to "Native xClC-5" Expression

As mentioned above, with the exception of ClC-0, nearly all ClC channels fail to adequately functionally express in *Xenopus* oocytes unless their 5′ and 3′ untranslated regions are removed or replaced. Our previously reported xClC-5 clone had a 5′ untranslated region (UTR) that contained a stretch of 33 additional nucleotides that were incorporated immediately before the ATG site. Apparently, the ATG codons within this region may have caused less efficient translation of the protein (Luneau et al., 1991; Swanson & Folander, 1992). In contrast, removal of the 5′ and 3′ UTR and replacement by 5′ and 3′ untranslated domains of the *Xenopus* β-globin gene lead to expression of this clone (Schmieder et al., 1998 and present study). This difference may reflect increased protein expression (Cribbs et al., 1990, Swanson & Folander, 1992) or increased stability of the xClC-5 mRNA or the efficiency of its translation (Krieg & Melton, 1984). Apparently, the expression of native xClC-5 clone does not incur these problems since this clone did not require the UTRs of the *Xenopus* β-globin gene for efficient expression, in contrast to the results of Schmieder et al. (1998) who used the ORF of the earlier xClC-5 clone (Lindenthal et al., 1997). Although several sequence differences exist between the open reading frame sequence of this earlier xClC-5 clone and "native" xClC-5, similar currents were observed by Schmieder et al. (1998) and in the present results. Therefore, these amino acid substitutions did not significantly alter the properties of the expressed currents.

External H^+ Inhibits ClC-5 Currents

Although some ClC channels are stimulated by reductions in extracellular pH (Rychkov et al., 1996, 1997; Jordt & Jentsch, 1997), acidic solutions inhibited the outward currents of ClC-5. For all three ClC-5 channel constructs, the inhibition of ClC-5 currents by protons occurred in a concentration-dependent manner with maximal effects on the outward currents at ~pH_o 5.3. For example, at pH_o values 7.5–9.5, no effects on xClC-5-ORF currents were found, whereas a slight stimulation was observed for hClC-5-ORF outward currents at pH values above 8.7. No effects of pH_o were detected on inward currents for xClC-5 or hClC-5. The half-maximal inhibition (calculated from a Hill plot of the effects of pH on the relative currents) was at a pH ~6 for both xClC-5 and hClC-5. Friedrich et al. (1999) have observed similar pH effects on hClC-5 and Schmieder et al. (1998) have also reported a pH inhibition of xClC-5. Our preliminary measurements of intracellular pH (Fong, Cooper & Wills, *unpublished*) indicate that intracellular pH in water-injected oocytes or xClC-5 RNA injected oocyte is not significantly affected by reduction of the external bathing solutions to pH 5.3. Therefore, the observed decrease in ClC-5 currents is unlikely to be mediated by changes in the intracellular pH.

Membrane Localization and Implications of pH Regulation of ClC-5

Unlike other well-characterized epithelial channels such as ENaC or CFTR, the normal function of ClC-5 channels and their location in renal cells has not been definitively established. On the basis of immunocytochemistry studies, Günther et al. (1998) and Luyckx et al. (1998) reported colocalization of ClC-5 proteins with H-APTase in intracellular vesicles below the brush border membrane in the proximal tubule. *In situ* hybridization studies by Obermüller et al. (1998) suggested that mRNA for ClC-5 was expressed only in the intercalated cells of the collecting ducts. Günther et al. (1998), but not Luyckx et al. (1998), found evidence for ClC-5 expression in α-intercalated cells of the collecting duct. The reasons for this discrepancy are unclear. It is also unknown whether trafficking of ClC-5 channels to the apical or basolateral membrane can occur. Although there is evidence that some intracellular ion transport proteins such as H^+ ATPase (*c.f.* Herak-Kaminberger et al. 1998) are vigorously recycled and trafficked between intracellular compartments and the apical or basolateral membranes under certain conditions, more information is needed concerning the site of expression of ClC-5 in renal epithelial cells and its potential trafficking between different membrane compartments.

Steinmeyer et al. (1995) and Friedrich et al. (1999) have proposed that ClC-5 channels might be facilitate the acidification of endosomes (e.g., as a route for chloride influx as a counterion to H^+ transport). If so, endosomal pH could be a potentially important inhibitor of ClC-5 channels located in endosomal membranes since the endosomal compartment pH ranges from 6.2–6.5 (Maxfield & Yamashiro, 1991). However, such an inhibitory regu-

lation by pH appears inconsistent with the above hypothesized role for ClC-5 channels. Moreover, if ClC-5 channels have similar rectification properties in endosomal membranes, they would be expected to mediate chloride efflux from the endosomal lumen, not influx. Further experiments are needed to resolve this paradox.

In the present study, the mechanism of inhibition of ClC-5 currents by acidic external solutions was not identified. At the macroscopic level, there were no detectable effects of pH on the gating of the currents. Similar results were reported by Friedrich et al. (1999) for human ClC-5. Therefore, any effects of pH on channel gating are either too rapid or complex to resolve, or the effects are mediated by other mechanisms. Although it is conceivable that alterations in membrane turnover such as reduced insertion or retrieval of channels from the membrane could occur, the rapid time course of the pH effect argues against this explanation. Alternatively, H^+ could interact with the channel either directly or indirectly at a site unrelated to the pore or with a regulatory protein. Further experiments at the single channel level are needed to resolve these issues. In preliminary experiments, we were unable to resolve detect single channel events for ClC-5. These results suggested that the single channel conductance of ClC-5 channels is low or that the channel opens infrequently.

In contrast to the inhibitory effects of external pH on ClC-5 outward currents, acidic external solutions activate inward currents of other ClC channels. For example, ClC-1 activity is stimulated due to a decrease in its inactivation by low pH_o (Rychkov et al., 1996). ClC-2 activity was also increased by acidic pH_o by a mechanism that shifted its voltage-gating to more positive potentials (Jordt & Jentsch, 1997). It would be interesting to see if any of these properties are due to direct effects on these channels. In contrast, extreme acidification transiently increased ClC-2 currents followed by inhibition (Furukawa et al., 1998). It is not known whether the subsequent inhibition effect was due to some unrelated secondary changes. As suggested by the data in Fig. 6, ClC-5 channels would be expected to be maximally active at a physiological pH of 7.5.

In summary, amphibian and mammalian ClC-5, when expressed in *Xenopus* oocytes, demonstrate similar large outwardly rectifying currents, a lack of sensitivity to chloride channel blockers, time-independence, a relative anion permeability sequence of $Cl^- \geq I^-$ and are reduced by acidic bathing solutions. The results indicate the potential usefulness of the A6 renal epithelial cell line as a tool for studying the trafficking and functional regulation of ClC-5 channels.

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