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Biochimica et Biophysica Acta 1566 (2002) 55–66

BIOCHIMICA ET BIOPHYSICA ACTA
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Review

Cloning and characterisation of amphibian ClC-3 and ClC-5 chloride channels

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Received 29 May 2002; received in revised form 19 July 2002; accepted 19 July 2002

Abstract

Amphibians have provided important model systems to study transepithelial transport, acid–base balance and cell volume regulation. Several families of chloride channels and transporters are involved in these functions. The purpose of this review is to report briefly on some of the characteristics of the chloride channels so far reported in amphibian epithelia, and to focus on recently cloned members of the ClC family and their possible physiological roles. The electrophysiological characterisation, distribution, localisation and possible functions are reviewed and compared to their mammalian orthologs.

Keywords: xClC-3; xClC-5; VSOAC; V-ATPase; Chloride channel; Amphibian; *Xenopus*

1. Introduction

Anion channels are present in all biological membranes, including plasma membranes and membranes of intracellular organelles (reviewed in Ref. [1]). Like all gated channels, they allow the passive diffusion of anions along their electrochemical gradients. Because chloride is the most abundant inorganic anion in the intra- and extracellular media, anion channels are commonly referred to as chloride channels. The gating properties of these channels are closely related to their function. Chloride channels are involved in various functions that may be housekeeping, or specialised and restricted to a particular tissue or cell type. Plasma membrane chloride channels play an important role in transepithelial transport involved in maintaining ionic homeostasis and acid–base balance. Plasma membrane chloride channels are also involved in cell volume regulation. This is particularly important for epithelial cells that are subjected to large and variable bulk flows. Another role of chloride channels at the plasma membrane is the control of membrane excitability, in particular in skeletal muscle, smooth muscle and neurons. Intracellular chloride channels are involved in setting the pH of different intracellular compartments, for instance along the endocytic pathway, in lysosomes and synaptic vesicles. However, the study of intracellular channels is technically more challenging and thus, little is known about their functional characteristics.

Amphibians are aquatic or semiaquatic animals that can adapt to live in water, which may vary from very low salt-containing water to brackish or even salty water [2]. Such adaptations are made possible through the specialisation of epithelia, including the skin, intestine, kidney, and urinary bladder allowing the control of the hydro-mineral and the acid–base balance of the internal medium of these animals. Amphibians have therefore provided important model systems to study transepithelial transport, acid–base balance, and cell volume regulation.

The purpose of this review is to describe the involvement of chloride channels in the above mentioned physiological functions that have been extensively studied in amphibian models, particularly adult frogs (*Rana esculenta*) and toads (*Bufo viridis* and *Xenopus laevis*). We focus particularly on

Abbreviations: cAMP, adenosine 2′,3′-cyclic monophosphate; PKA, protein kinase A; PKC, protein kinase C; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; NPPB, 5-nitro-2-(3-phenylpropylamino)-benzoic acid; DPC, diphenylamine-2-carbonic acid; 9-AC, anthracene-9-carboxylic acid

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PII: S0005-2736(02)00594-1

the distribution, cellular localisation, and functional characterisation of two amphibian chloride channels: xClC-5 and xClC-3.

2. Transepithelial chloride transport, ionic homeostasis, and acid–base balance

The frog skin has been extensively used as a model to study Na^+ transport in epithelia. Cells of the outermost cell layer possess tight junctions [3] and communicate with the deeper cell layers by gap junctions [4]. This cell layer is composed of two main ion transporting cell types, principal cells and mitochondria-rich cells (MR cells) [5]. Due to its cellular and functional organisation, this highly polarised epithelium was proposed to behave as a syncitium [6,7]. The "Ussing model" has drawn the fundamental lines of our understanding concerning active salt absorption in tight epithelia [6]. The short-circuited skin bathed on both sides with a Ringer solution and mounted in a Ussing chamber has been a key tool in this context. In the process of active salt absorption, Na^+ is considered as a major player because it is the sole actively transported ion. Chloride ions have played the secondary role of an "accompanying anion" maintaining the electroneutrality of ion transfer. However, a large chloride transport (and conductance) occurs through skins bathed in high NaCl-containing media under open circuited conditions or imposed voltages similar to physiological transepithelial potentials [8–10]. This passive chloride conductance, activated by voltage and by high chloride concentrations is regulated by second messengers and hormones; this phenomenon has been particularly studied in toads (reviews in Refs. [11,12]). Patch-clamp experiments allowed the identification of chloride channels that mediate CFTR-like currents in MR cells, suggesting that these cells are responsible for the transepithelial chloride conductance (Refs. [13,14]; Larsen, this issue). The existence of a chloride conductance in MR cells is in agreement with the early finding of a positive correlation between the number of MR cells and the transepithelial Cl^- conductance [15]. Nevertheless, the existence of a paracellular pathway for Cl^- has also been implicated [16], and the relative contribution of each pathway is still not solved (Refs. [11,12]; Nagel and Larsen, this issue). The physiological significance of this transepithelial chloride conductance remains to be clarified in toads as well as in frogs where it has also been reported [17].

Most amphibians that live in freshwater have to face a low NaCl concentration (<1 mM) in the ambient medium. Therefore, the reabsorption of Na^+ and of Cl^- is active and requires complex transport mechanisms. The reabsorption of Na^+ and Cl^- is achieved by separate and independent anion- and cation-transporting mechanisms initially described in vivo [18–22], and later in vitro [10,23,24]. The rate of net absorption of sodium and chloride can be different, depending on the hydro-mineral and acid–base status of the animals [25], thus demonstrating a fine regulatory process. In agreement with the Ussing model, sodium ions diffuse through amiloride-sensitive sodium channels [26,27] located on the apical membranes of principal cells and of MR cells [25,28]. Na^+ ions are then pumped into the "milieu intérieur" by the Na^+ pump located on the basal membranes. The electrochemical driving force for the apical Na^+ entry depends mainly on the activity of an electrogenic V-ATPase, which provides the negative cell potential that compensates for the unfavourable chemical gradient [29–31]. Therefore, an indirect electrical coupling links the proton pump to sodium entry and accounts for the correlation between sodium absorption and proton secretion. In this scheme of Na^+ reabsorption from very dilute solutions, two different pumps functioning in series are required: the H^+ pump and the Na^+ pump. This frog skin model in which MR cells play a key role has been extended to freshwater fishes [32,33] and salt-depleted crustaceans [34].

The active chloride absorption that parallels reabsorption of sodium from low salt containing media, is electrically coupled to the proton pump and participates in the regulation of ion homeostasis and the acid–base balance of the organism. In MR cells, the electric gradient provided by the V-ATPase energises the electroneutral Cl^-/HCO_3^- exchanger at the apical membrane [35]. Thus, absorbed Cl^- ions are exchanged for secreted bicarbonate [24,36]. This secondary active mechanism presents an apparent affinity for chloride of 0.2 mM in *R. esculenta* frog skin [9]. In isolated skins of amphibians bathed with isotonic Ringer solutions, this exchange mechanism still exists, but allows only a small portion of chloride transport compared to conductive chloride absorption [9,11].

A model illustrating the key roles of MR cells and of V-ATPase in energising chloride and sodium transport in frog skin is given in Fig. 1.

3. Anion channels and cell volume regulation

Many cell functions such as epithelial transport, cell proliferation, and apoptosis require cell volume regulatory mechanisms (for a review, see Ref. [37]). Anion channels have been implicated in cell volume regulation following cell swelling (also called regulatory volume decrease or RVD). Cell swelling is caused by acute changes in medium osmolarity (hyposmotic shocks) or by metabolic changes. The recovery (shrinkage) back to the initial cell volume is achieved by the opening of potassium and anion channels that in turn drive water exit. Several different anion channels, distinguishable by their electrophysiological characteristics, have been reported in amphibian kidney cells upon an hyposmotic challenge [38], but one channel type is reported in most models studied, the so called VSOAC for volume sensitive organic osmolyte and anion channel (also called volume regulated anion channel, VRAC). This channel presents an outwardly rectified anion conductance, a char-

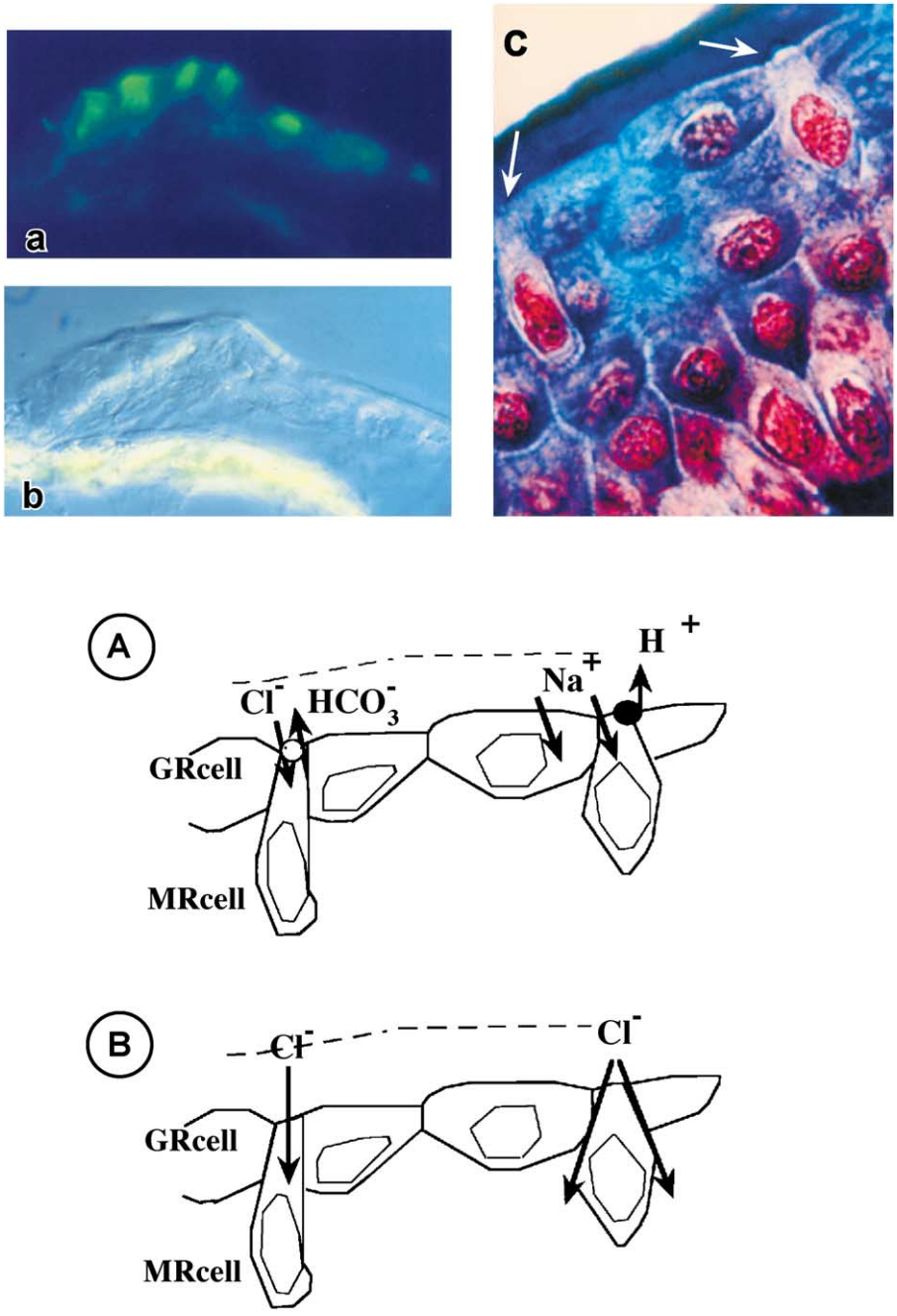

Fig. 1. V-ATPase in MR cells energises ion transport in frog skin. Upper part: V-ATPase immunoreactivity of MR cells in *R. esculenta* skin epithelium viewed by epifluorescence illumination (a), the same view by differential interference contrast is shown in (b) (from Ref. [11]). The frog skin epithelium presents different cell layers (stratum corneum, stratum granulosum, stratum spinosum, and basal lamina). Two MR cells are clearly distinguishable in the outermost cell layer (c). Lower part: (A) Model of ion reabsorption from low salt containing media. The electrogenic proton pump (V-ATPase) in MR cells energizes the Na^+ entry (through amiloride sensitive sodium channels present in granular (GR) and MR cells) and the Cl^-/HCO_3^- exchanger present in the same MR cells [11,35]. (B) Model of ion reabsorption in isotonic saline. A large chloride conductance is present in high salt containing solutions; the relative contribution of the cellular pathway through MR cells [11] and a paracellular pathway [12] is not solved. For clarity, the Cl^-/HCO_3^- exchanger (A) is not represented here; its contribution to chloride absorption is smaller than that of the chloride conductance, but it is nevertheless significant [9,11].

acteristic pharmacology and anion selectivity. In addition, it is permeable to small organic osmolytes including taurine, *myo*-inositol or sorbitol (for reviews, see Refs. [39,40]). Amphibian epithelial models used to study cell volume regulation include frog skin (in particular MR cells) [41,42], renal cells [43,44], and urinary bladder cells [45]. In addition to these epithelial cells, *Xenopus* oocytes have been particularly useful to study the cell volume activated chloride conductance present in manually defolliculated oocytes [46]. A comparison of the electrophysiological characteristics of the volume-sensitive anion conductance in *Xenopus* oocytes and A6 renal cells is given in Table 1. In

Table 1
Comparison of the swelling-activated chloride current and taurine effluxes in two *Xenopus* model cells: oocytes and renal cells

	Xenopus A6 (renal cell line)		*Xenopus* oocytes	
	^{3}H-taurine efflux	Swelling-activated chloride current	^{3}H-taurine efflux	Swelling-activated chloride current ($I_{Clswell}$)
Osmotic stimulation	Yes[a]	Yes[a,b,c]	Yes[a]	Yes[d,e]
Outward rectification		Yes[a,b,c]		Yes[d,e]
Localisation	Basolateral membranes[a]	Basolateral membranes[d]		
Pharmacology				
DIDS	Blocks[a]	Blocks[a]	Blocks[a]	Blocks[d,e]
Oxonol	Blocks[a]	Blocks[a]	Blocks[a]	Blocks[d]
NPPB blocks	Blocks[a]	Blocks[b]	Blocks[a]	Blocks[d,e]
Ketoconazole	Blocks[a]		No effect[a]	Slightly blocks[d]
Extracellular nucleotide	Stimulates[a]	?	Blocks[a]	Blocks[d,e]
Lanthanum	?	?		Blocks[d,e]

[a] Our unpublished data.
[b] From Ref. [105].
[c] From Ref. [38].
[d] From Ref. [66].
[e] From Ref. [46].

these two amphibian models, the volume-sensitive anion conductances present similar levels of outward rectification and similar sensitivities to the pharmacology (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), oxonol, and 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) blockade). Osmo-sensitive taurine effluxes are also blocked by these inhibitors and the IC_{50} (5 μM) of oxonol (one of the most effective blockers) in A6 cells compares to that found for RVD (mediated by Cl^- and K^+ exit), suggesting the presence of a common pathway for chloride and taurine (unpublished data). However, the discrepancies in the effects observed with ketoconazole and extracellular nucleotides could be explained by the existence of multiple osmo-sensitive chloride channels [38]. Even though our knowledge of the properties of the volume-sensitive anion conductance and the signalling pathways involved in its regulation have progressed this past decade (for reviews, see Refs. [40,47]), the molecular identity of the channel protein is still unknown.

4. Molecular characterisation of amphibian chloride channels of the ClC family

Chloride channels are encoded by at least three different gene families. The largest is the gene family that encodes the pentameric ligand-gated chloride channels [1,48,49]. The ClC gene family encodes voltage-dependent chloride channels that are thought to function as dimers and may require additional β subunits to function [1]. The cystic fibrosis transmembrane conductance regulator (CFTR) belongs to the ABC transporter gene family, but is so far the only one in this family that functions as a chloride channel and also as a channel regulator [1,50,51]. Another emerging chloride channel gene family is that of the CLCA family, which is thought to encode calcium-activated chloride channels (also referred to as CaCC) [1,52,53]. However, more chloride channel genes remain to be discovered, such as swelling activated chloride channels (and possibly CaCC). Also, not all identified chloride channels have been attributed a function. The difficulties in correlating a chloride channel with a particular function result from the lack of specificity of the pharmacology for a broad diversity of chloride channels. In addition, the study of chloride channel function is hampered by the presence of endogenous chloride channels in virtually every cell system used for functional expression.

To date, only four chloride channels have been cloned from amphibians: the *Xenopus* homologue of CFTR [54], the *Xenopus* homologue of ClC-K [55], and the *Xenopus* homologues of ClC-5 and ClC-3, which were identified in our laboratory (Ref. [56]; our unpublished data), by the following strategy.

A rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR) cloning strategy was carried out to isolate amphibian homologues of the ClC gene family from A6 renal cells. Amplifications were based on degenerate oligonucleotide primers designed from consensus sequences of the *Torpedo* ClC-0 and the mammalian ClC-1, -2 and -K sequences published at the time of the beginning of our experiments. We identified two novel sequences of 2586 bp (a) and of 3016 bp (b), both showing significant similarity to ClC genes. A translation product of 791 amino acids with a molecular mass of 88 kDa is predicted from the open reading frame (ORF, nucleotides 91–2464) from the first sequence (a) when the initiation methionine is assigned to the first ATG codon in-frame. A 808-amino acid translation product with a molecular mass of 90 kDa can be deduced from the second sequence (b). An amino acid identity of 69% is found between the two sequences. A database search using the BLASTN program revealed extremely high sim-

ilarities of the isolated sequences to other sequences of ClC proteins identified meanwhile. Sequences (a) and (b) were identified as the amphibian orthologs of mammalian ClC-3 and ClC-5, respectively. Therefore, the new sequences were termed xClC-3 and xClC-5 (where "x" stands for *Xenopus*). Amphibian ClC-3 protein displays 89% identity to human ClC-3 [57], 90% to rat and mouse ClC-3 [57,58], 90% to guinea pig ClC-3 [59] and 86% to teleost fish ClC-3 [60]. *Xenopus* ClC-5 protein has 78% identity to its human and rat orthologs [61,62]. If the 5th methionine of the open reading frame is considered as the initiation ATG, the identity to the mammalian orthologs increases to 85%, respectively. The two polypeptides both display the typical primary structure of members of the ClC family, i.e. containing the characteristic 13 hydrophobic domains. As most ClC channels, both amphibian ClC amino acid sequences show a highly conserved potential N-glycosylation site located between hydrophobic regions D8 and D9. Several motifs for potential phosphorylation by protein kinase C (PKC) and phosphorylation by cAMP- and guanosine 3′,5′-cyclic monophosphate-dependent protein kinase are present in both sequences.

5. *Xenopus* oocytes: expression system of choice for electrophysiological characterisation of channel proteins

The *Xenopus* oocyte is the expression system of choice for electrophysiological studies of channel proteins and transporters. A large number of oocytes can be easily obtained from a single female and the large size (about 1.2–1.3 mm in diameter) of the oocytes allows the microinjection of mRNA of the protein(s) of interest followed by voltage-clamp analysis using double microelectrodes. The oocytes can also be used for biochemical studies to assess protein expression, localisation, and posttranslational modifications. However, the proteome of the amphibian oocyte does not necessarily include accessory and regulatory proteins required for proper functioning, trafficking, or regulation of the exogenously expressed proteins. Moreover, *Xenopus* oocytes express endogenous channel proteins, which can be activated under various circumstances and contribute to measured currents or even interfere with the activity of exogenous proteins. Some endogenous channel activities are well known, such as the transient calcium-activated chloride current initially described by Miledi and Parker [63], or the hyposmotically activated chloride current $I_{Cl\ swell}$, which appears only in manually defolliculated oocytes [46]. Another constitutive chloride current that is activated by an unknown mechanism has been described in several studies [64–66]. Among the endogenous chloride channel proteins, xClC-3 and xClC-5 are the only ones that have been characterised at the molecular level (Ref. [56]; our unpublished results). Although high levels of ClC-3 and ClC-5 mRNA have been found, only small amounts of these proteins are synthesised. To date, these channels have not been described to interact with exogenous proteins, in particular ClCs. Even though ClC channels have been described to form multimers with novel properties [67], the electrophysiological properties of ClCs appeared the same when they were studied in mammalian cell systems [68,69]. The absence of interaction of endogenous ClCs with exogenous ClCs is probably due to their low expression level and their likely intracellular localisation in the oocyte.

6. Functional characterisation of amphibian chloride channels of the ClC family

6.1. xClC-3

6.1.1. Electrophysiological characterisation

Expression of xClC-3 as well as of ClC-3 orthologs in *Xenopus* oocytes or mammalian cell systems did not yield any novel currents in the hands of several groups (Ref. [57,62,68,70]; our unpublished results; Fig. 2). The absence of ClC-3 currents could be due to a lack of regulatory elements or a β subunit. Barttin has recently been identified as a β subunit necessary for ClC-K channel function [71]. However, it is more likely to be due to the intracellular localisation of the channel. Nevertheless, several other groups reported functional expression of ClC-3. The first reports of ClC-3 expression by Kawasaki et al. [58,72] describe currents with divergent characteristics found in different expression systems and an $I^->Cl^-$ selectivity that is not consistent with the selectivity observed with other ClC channels. Duan et al. [59] reported that expression of ClC-3 yielded swelling-activated currents with a $I^->Cl^-$ selectivity and demonstrated specific effects in the current characteristics with several point mutations [73,74]. Finally, ClC-3 currents with electrophysiological characteristics resembling the closely related ClC-4 and ClC-5 channels (rectification, insensitivity to DIDS or hyposmotic stress, and $Cl^->I^-$) have been recently reported in CHO-K1 cells [75]. The divergence of these reports and of the current characteristics associated with ClC-3 in different expression systems is probably related to the difficulty to distinguish between endogenous currents and expressed currents.

6.1.2. Distribution, localisation, and functional model

RNase protection assays (RPA) have shown that xClC-3 is a rather broadly expressed gene in *X. laevis*. A high expression level was found in the central nervous system, and significant levels of expression were also observed in kidney, intestine, skeletal muscle and lung (S. Lindenthal, unpublished data). In rodent, a broad tissue distribution of ClC-3 was reported at the mRNA level [58] and at the protein level [76,77], with highest expression levels in brain.

Polyclonal antibodies against a C-terminal peptide of xClC-3 were raised. On Western blots with membranes of xClC-3 expressing *Xenopus* oocytes, a broad band at 105

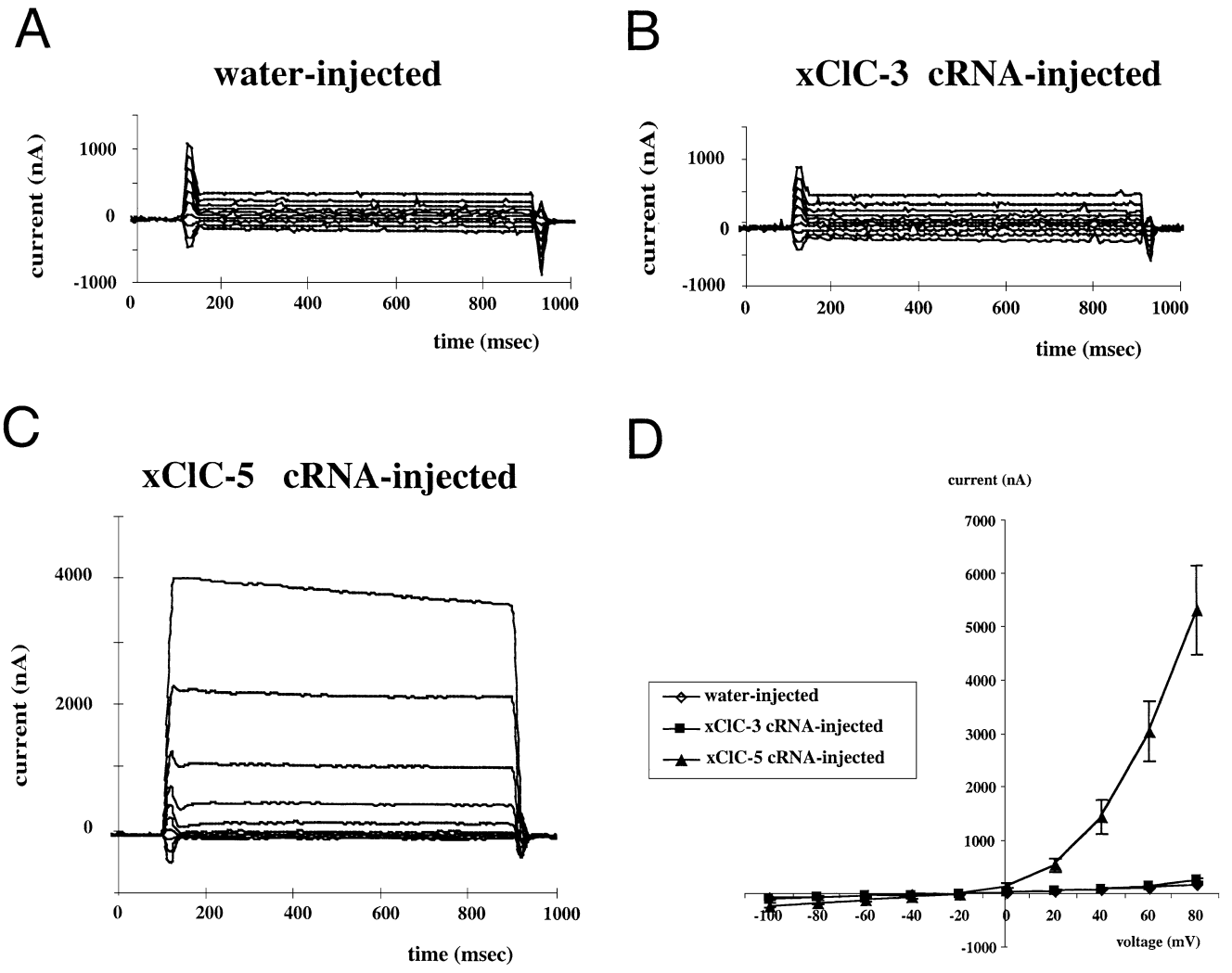

Fig. 2. Electrophysiological properties of I_{xClC-3} and I_{xClC-5}. Representative traces of currents of (A) water-, (B) xClC-3 cRNA- and (C) xClC-5 cRNA-injected oocytes. The oocytes were investigated by voltage-clamp 4 days after injection of 5 ng cRNA/oocyte or water. Oocytes were sequentially clamped from a holding potential of −50 mV to voltages between −100 and +80 mV for 800 ms in steps of 20 mV. (D) Mean current–voltage (I/V) relationships of water-injected oocytes (♦, $n=17$), xClC-3 cRNA-injected oocytes (■, $n=17$) and xClC-5 cRNA-injected oocytes (▲, $n=15$). Data from Ref. [66] and our unpublished data. Methods are as described in Ref. [66].

kDa was revealed (Fig. 3A). After enzymatic deglycosylation, xClC-3 protein migrated as a lower molecular mass band of about 85 kDa close to its calculated molecular mass of 88 kDa. Endogenous xClC-3 expression in A6 cells and in *Xenopus* kidney was also analysed on immunoblots. Similar to xClC-3 expressed in *Xenopus* oocytes, endogenous ClC-3 in A6 cells is highly glycosylated and migrates at a molecular mass of 105 kDa (Fig. 3B). In mouse, mClC-3 is also highly glycosylated and was found to undergo differential tissue-specific N-glycosylation [77].

Localisation of endogenous xClC-3 in A6 cells by immunocytochemistry shows extensive intracellular staining (our unpublished observations). This localisation is consistent with recent studies showing the presence of mammalian ClC-3 in endosomes and synaptic vesicles [76]. In ClC-3 knock-out mice, acidification of synaptic vesicles was found to be impaired, and led the authors to propose that the ClC-3 conductance may function as an electrical shunt for the proton pump (V-ATPase) to facilitate intravesicular acidification [76]. Labeling of cell surface proteins showed that ClC-3 can be found at the plasma membrane under some conditions of overexpression [70]. Whether endogenous ClC-3 protein can also reach the plasma membrane remains to be examined. Recently, a splice variant of ClC-3, called ClC-3B, has been cloned from human pancreas [78]. This ClC-3B variant is expressed mainly in epithelial cells, and interacts with the PDZ-domain protein EBP50 (also called NHERF1), which is also known to interact with CFTR [79]. Interestingly, co-transfection of ClC-3B and EBP50 induced the activity of the outwardly rectifying chloride channel (ORCC) at the leading edges of the cells. This led the authors to propose that ClC-3B may function as the CFTR-regulated ORCC or as a regulator of ORCC (through the regulation of ORCC

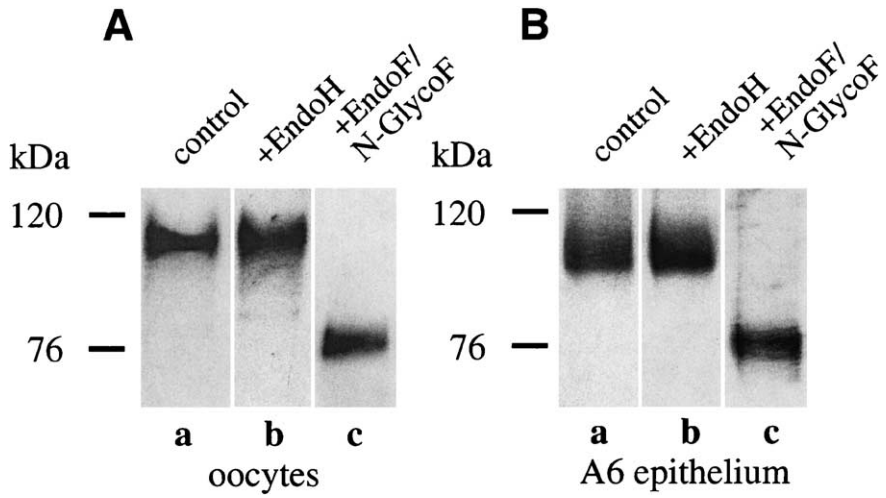

Fig. 3. Western blot analysis of xClC-3 from cRNA-injected *Xenopus* oocytes and A6 cells, and enzymatic deglycosylations. Membrane preparations obtained from xClC-3 cRNA-injected oocytes (A) and A6 cells (B) were incubated at 37 °C for 2 h without ("control", lane a) and with endoglycosidase H ("+EndoH", lane b) or endoglycosidase F/N-glycosidase F mixture ("+EndoF/N-GlycoF", lane c) prior to separation on SDS/PAGE and immunoblotting. Endoglycosidase H digestion had no effect on xClC-3 from injected oocytes and A6 cells. For both samples, digestion with the endoglycosidase F/N-glycosidase F enzyme mixture led to a shift of the recognised band from 105 to 85 kDa corresponding to the calculated molecular mass of unglycosylated xClC-3 protein. Methods are as described in Ref. [77]. Expression of xClC-3 was carried out according to the methods described for expression of xClC-5 [66].

trafficking) [78]. However, it is not yet clear whether ClC-3B channels reach the plasma membrane when transfected into C127 cells.

The xClC-3 cDNA that we identified is similar to ClC-3A [78], but the presence of a ClC-3 splice variant (xClC-3B) in amphibian chloride secreting epithelia (as A6 cells) remains a possibility that requires further examination. Future studies to elucidate the function of ClC-3 splice variants in β-intercalated cells in rat kidney [80], as well as in fish and amphibian intestines (our unpublished observations), are also required.

6.2. xClC-5

6.2.1. Electrophysiological characterisation

To date, ClC-5 orthologs from human, rat, mouse, pig, guinea-pig, and *Xenopus* have been functionally expressed in either *Xenopus* oocytes or mammalian cell lines (HEK 293, COS-7, and CHO-K1) (Table 2). The functional characteristics of the amphibian xClC-5 have been studied in *Xenopus* oocytes and COS-7 cells. In both systems, the currents associated with xClC-5 expression, $I_{ClC\text{-}5}$ appeared voltage dependent. The current–voltage relationship of $I_{ClC\text{-}5}$ shows a strong outward rectification, with significant currents only for membrane potentials higher than +20 mV (Fig. 2). The currents observed with ClC-5 orthologs are equally outward rectified. The physiologic relevance of such a strong voltage-dependence is not understood yet. ClC-5 has been localised in early endosomes [81]. The membrane potential of this intracellular compartment is not documented and it is therefore not known whether the membrane potential would allow ClC-5 activity. Also, the association with some regulatory factor or β subunit might shift the voltage-dependence of the current towards more physiologic potentials.

Due to the strong outward rectification of the current, it is not possible to define the relative anion permeability sequence. Therefore, only the conductivity sequence at positive membrane potentials could be determined. We deter-

Table 2
Comparison of electrophysiological characterisations of CIC-5 orthologs

Species	Expression system	Rectification	Conductivity sequence	pH sensitivity[a]	Pharmacology[b]	Reference
Rat	*Xenopus* oocytes	strong outward	NO_3^->Cl^->Br^->I^-≫glutamate	–	no effects	[62]
Xenopus	*Xenopus* oocytes	strong outward	NO_3^->Br^->Cl^->I^-≫gluconate	yes	no effects	[66]
Human	*Xenopus* oocytes	strong outward	–	yes	–	[68]
Human	HEK 293	strong outward	–	yes	–	[68]
Xenopus	*Xenopus* oocytes	strong outward	NO_3^->Cl^-=I^->bicarb≫glutamate	yes	no effects	[69]
Human	*Xenopus* oocytes	strong outward	NO_3^->Cl^->bicarb>I^-≫glutamate	yes	no effects	[69]
Pig	*Xenopus* oocytes	strong outward	NO_3^->Cl^->Br^->acetate>I^-≫gluconate	yes	no effects	[88]
Xenopus	COS-7	strong outward	Cl^->I^-	yes	–	[82]
Guinea pig	HEK 293	strong outward	NO_3^->Cl^->Br^->I^->F^-≫gluconate	–	–	[106]
Mouse	CHO-K1	strong outward	–	–	no effects	[107]

[a] Inhibition by acidic extracellular medium.
[b] Commonly used chloride channel inhibitors (DIDS, NPPB, DPC).

mined a conductivity sequence of NO_3^->Br^->Cl^->I^-≫ gluconate for xClC-5 expressed in *Xenopus* oocytes [66]. The preference of Cl^- over I^- is in agreement with the conductivity sequences found for ClC-5 orthologs. Moreover, it is a conserved feature of all members of the ClC family and allows to distinguish the ClC currents from endogenous currents that present a conductivity sequence of I^->Cl^-. For an unknown reason, Mo et al. [69] found similar conductances for Cl^- and I^- for xClC-5 in oocytes. In COS-7 cells, however, they also found a conductivity sequence of Cl^->I^- [82]. ClC-5 has been localised in early endosomes in several cell types [81,83] and has been proposed to function in the electroneutral acidification of endosomes by the V-type proton pump (see Fig. 5C). Interestingly, the acidification of endosomes from the proximal tubule can be stimulated by anions, with Cl^- being more effective than I^- [84].

Another characteristic that is conserved among ClC-5 orthologs is the dependence on the pH of the extracellular medium. In our study, xClC-5 was not sensitive to increases in the pH of the extracellular bathing medium. However, lowering the pH was found to reversibly inhibit the xClC-5 current with a pK_a value of 5.67 and a Hill coefficient of 2.2, which is consistent with the likely dimeric structure of the ClC channel [85–87]. Other reports on the pH sensitivity of ClC-5 from *Xenopus* or other species are in agreement with our finding [68,69,82,88], with the exception of the determination of the Hill coefficient in two reports that gave values equal or close to 1 [69,88]. Given the intracellular localisation of ClC-5, its inhibition by low extracellular pH is likely to play a physiologically important role. Indeed, the luminal space of an intracellular compartment can topologically be considered equivalent to the extracellular space. Therefore, the inhibition of ClC-5 conductance by an acidic luminal pH would be consistent with a negative feedback mechanism that allows to set the luminal pH of the intracellular compartment, i.e. the endosomes in the proximal tubule.

Classic anion channel inhibitors, such as DIDS, NBBP, and anthracene-9-carboxylic acid (9-AC) have been without any effect on ClC-5 currents. We also used various other pharmacological agents that have been described to block anion currents (oxonol, riluzole, niflumic acid, ketoconazole, tamoxifen, verapamil, gossypol, diphenylamine-2-carbonic acid (DPC), cAMP, lanthanum) on xClC-5 currents, but still no inhibition was observed [66]. Thus, ClC-5 appears completely insensitive to the pharmacological substances commonly used to characterise anion conductances. However, Weng et al. [82] recently reported a significant and reversible inhibition of the amphibian ClC-5 in the presence of 100 μM H_2O_2 when expressed in COS-7 cells. In *Xenopus* oocytes, however, only a high concentration (10 mM) of H_2O_2 or long incubation times (1 mM for 20 h) were effective at xClC-5 inhibition. The physiological relevance of this inhibitory effect is unknown.

The amino acid sequences of ClC-5 proteins predict several conserved putative phosphorylation sites for protein kinase A (PKA) and PKC. Several groups therefore attempted to inhibit the ClC-5 conductance with PKC inhibitors (Ref. [82]; our unpublished data) or to stimulate the conductance by increasing the intracellular level of cAMP (Ref. [62,82]; our unpublished data). Both approaches were without success but Weng et al. [82] reported the inhibition of the xClC-5 conductance by 10 μM H-89, a potential inhibitor of PKA. Whether other ClC-5 orthologs are also inhibited by H-89 and whether this inhibition is mediated through PKA remains to be determined. Interestingly, the chloride conductance involved in the acidification of proximal tubule endosomes has been described to be regulated by PKA [89].

We also investigated the effect of several tyrosine kinase inhibitors on xClC-5 expressed in *Xenopus* oocytes (our unpublished results). Geldanamycin and cinnamic acid had no effect on xClC-5 currents. However, genistein another potent tyrosine kinase inhibitor was found to inhibit the current significantly and reversibly (Fig. 4). Daidzein, the inactive structural analog of genistein did not produce this inhibitory effect on ClC-5, indicating that genistein does not

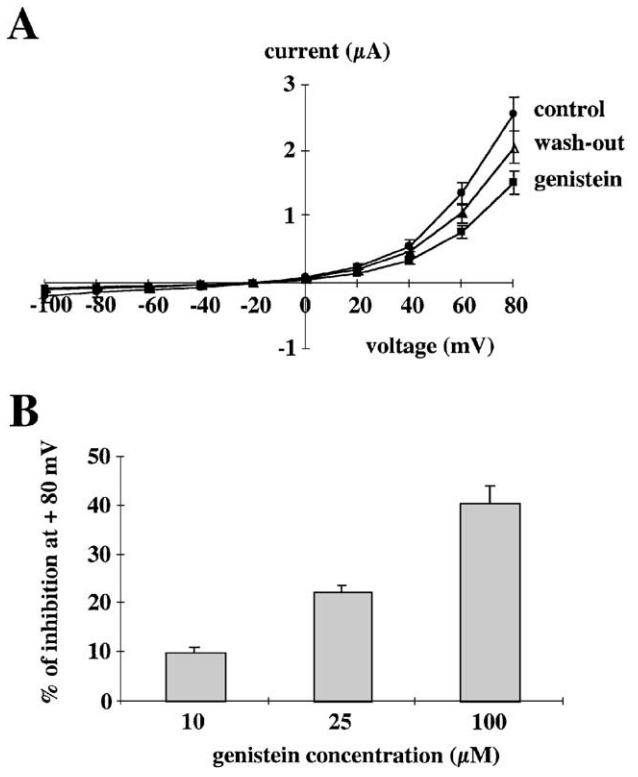

Fig. 4. xClC-5 current is inhibited by the tyrosine kinase inhibitor genistein. (A) Current/voltage relationships of oocytes expressing xClC-5 under control conditions, after 5 min perfusion with 100 μM genistein, and after 5 min of wash-out of the inhibitor. An inhibition of 41 ± 2% was achieved after 5 min of perfusion with genistein. Only 50% of this inhibition could be recovered after a 5 min wash-out period. Daidzein as a control was ineffective (not shown). (B) Inhibition of xClC-5 by 10, 25 and 100 μM genistein. Represented are the percentage of inhibition of the currents measured at + 80 mV (our unpublished data). Methods for cRNA injection and electrical recordings are as described in Ref. [66].

directly interact with ClC-5. In addition, tyrphostin 51, yet another tyrosine kinase inhibitor, was also able to reduce the ClC-5 conductance. These inhibitory effects of genistein and tyrphostin 51 could also be observed on the human ClC-5. Further work is needed to investigate the regulation of ClC-5 by tyrosine kinases.

6.2.2. Distribution

In *Xenopus*, RPA have shown that xClC-5 is a rather broadly expressed gene with highest transcription levels in oocytes, kidney, and intestine [56]. xClC-5 is also present in liver, blood, brain, heart, and urinary bladder. We also examined the tissue distribution in *X. laevis* by Western blot using a polyclonal antibody against the 16 C-terminal amino acids of the xClC-5 protein. High expression levels were found in kidney and intestine, and much lower levels in brain and heart. This distribution was confirmed with a different antibody directed against the N-terminal region of rClC-5 (kindly provided by T. Jentsch) (Fig. 5A). High ClC-5 mRNA levels were also reported in kidney, intestine and gill of the teleost fish, *Oreochromis mossambicus* [60]. In contrast, in rodents and human, the ClC-5 expression levels are elevated only in kidney [81,90], and much lower in intestine [83]. Thus, the expression of ClC-5 seems to be more restricted in higher vertebrates then in lower vertebrates like *Xenopus* and *Oreochromis*.

6.2.3. Localisation and functional model

We examined the subcellular localisation of ClC-5 in A6 cells by immunofluorescence (Fig. 5B, our unpublished data). As expected, xClC-5 protein was abundantly expressed in this cell line derived from *Xenopus* distal tubule. The protein was localised in intracellular compartments throughout the cytosol and concentrated in the perinuclear region. In *O. mossambicus*, it has also been established that ClC-5 functions as an intracellular channel [60].

In rodent kidney, ClC-5 was found in the proximal and collecting tubule, as well as in the thick ascending limb of Henle's loop [81,90–92]. In rat proximal tubule, ClC-5 was localised predominantly in cytoplasmic vesicles below the brush border where it co-localises with the proton pump [81,90] and with fluorescently labelled endocytosed proteins

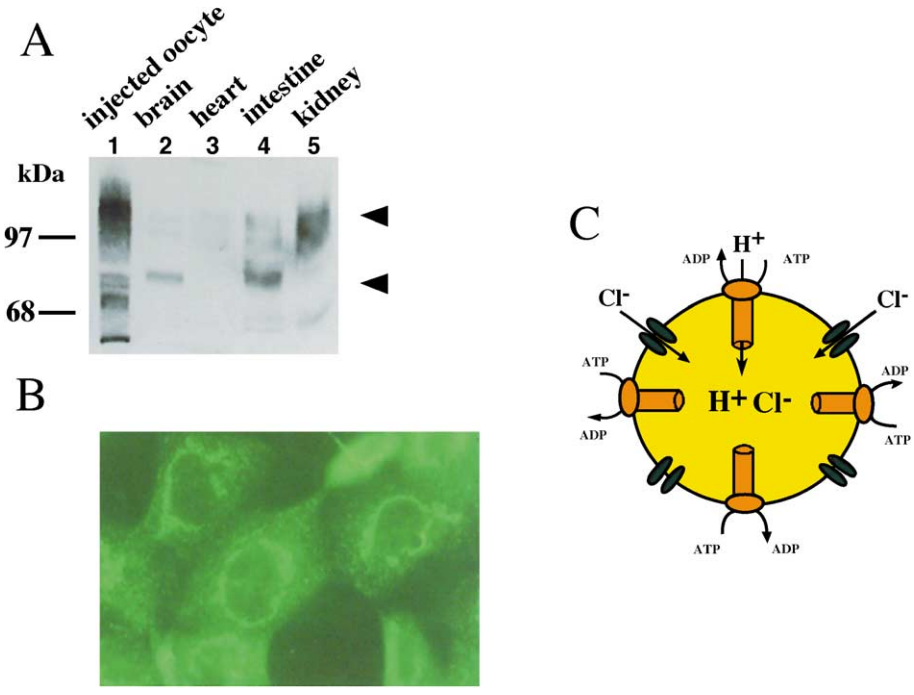

Fig. 5. Tissue distribution and localisation of xClC-5. (A) The tissue distribution of xClC-5 was examined in *Xenopus* with anti-ClC-5 antibodies described in Ref. [81]. Bands of different molecular weights could be detected in xClC-5 cRNA-injected oocytes, kidney, intestine, and brain. The highest band (130 kDa, arrowhead) was predominant in cRNA-injected oocytes and kidney and is likely to correspond to a highly glycosylated form of xClC-5. A faint band was seen in heart. Brain and intestine presented a predominant lower band (at about 85 kDa, arrowhead). The same pattern of bands was observed with our antibody (antibody described in Ref. [66]). The 50 kDa band observed in oocytes corresponds probably to a nonspecific band as it can also be observed in noninjected control oocytes (data not shown). Methods are as described in Ref. [66]. Briefly, 50 μg of *Xenopus* oocytes and 150 μg of *Xenopus* tissues were separated by SDS-PAGE, electrotransfered onto nitrocellulose membrane, and incubated overnight with the anti-ClC-5 antibodies, kindly provided by T. Jentsch. (Our unpublished data.) (B) Immunolocalisation of xClC-5 in A6 cells grown on coverslips. Cells were fixed in 2% paraformaldehyde. Primary anti-ClC-5 antibodies were described previously [66] and used at 1:50. Secondary antibodies were FITC-conjugated, and were used at 1:300. Bar: 10 μm. (Our unpublished data.) (C) Functional model for ClC-5 (model modified from Ref. [104]). ClC-5 co-localises with proton pumps in early endosomes in proximal tubule cells [104]. Parallel functioning of the proton pumps and ClC-5 channels allows the acidification of early endosomes involved in recycling and degradation of apical receptors and reabsorption of low molecular weight proteins.

[81]. Staining of the brush border was also reported [90]. The co-localisation of ClC-5 with proton pumps in endosomes led the authors [81] to propose that ClC-5 may function in parallel in the acidification of endosomes, by providing the electrical shunt required for proper functioning of the V-ATPase (Fig. 5C). Subsequently, this hypothesis was further supported by a knock-out mouse model in which the ClC-5 gene was disrupted [93]. In these mice, fluid-phase and receptor-mediated endocytosis were impaired, as a consequence of ClC-5 disruption. ClC-5 is also expressed in α- and β-intercalated cells of the collecting duct that are involved in acid and base excretion, respectively [81,90]. In α-intercalated cells, ClC-5 was found to co-localise with the proton pumps in apical vesicles, and was suggested to be a key element for endosome acidification and proton secretion into the lumen [90].

By analogy with the proposed model for ClC-5 function in endosomes, it is tempting to speculate about ClC-5 function in the frog skin epithelium. One could imagine ClC-5 functioning in parallel to the V-ATPases in the "pit" of MR cells (Fig. 1). Acidosis modulates proton secretion and the rate of vesicle exocytosis in the frog skin epithelium [25,94]. Thus, acidosis could trigger the exocytosis of ClC-5 containing vesicles, providing a regulatory mechanism to control V-ATPase activity (proton excretion). Immunocytochemical studies to localise xClC-5 in the proton secreting frog skin epithelia and functional studies would be necessary to test this hypothetical model for ClC-5 function in the frog skin.

6.3. xClC-K

A third member of the ClC family has been cloned from *Xenopus* [55]. Comparison with other members of the *ClC* gene family showed that the sequence is most closely related to the ClC-K subbranch. It shares 60–62% similarity with its rat and human orthologs, respectively, and has been named xClC-K. This cDNA encodes a 77 kDa protein presenting about 30% similarity with *Xenopus* ClC-3 and ClC-5. ClC-Ka and ClC-Kb channels represent two closely related members (approximately 90% identity) within the ClC gene family [95–97]. Both channels are predominantly expressed in the kidney [95–97], but are also present in the inner ear [71,98]. Recently, barttin, a small protein with two transmembrane domains, has been identified as a β subunit for ClC-K channels, which is necessary for channel activity [71]. There is strong evidence that the ClC-K/barttin heteromers play an important role in transepithelial transport in the kidney and the stria vascularis. In human, mutations in ClC-Ka lead to nephrogenic diabetis insipidus [99], suggesting that ClC-Ka may be involved in the chloride transport in the thin ascending loop of Henle. ClC-Kb was found to mediate the basolateral chloride efflux in the thick ascending limb of Henle's loop and mutations of ClC-Kb are responsible for Bartter's syndrome [100]. The role of ClC-K in *Xenopus* kidney remains to be determined.

6.4. Other ClC family members

Five additional *ClC* genes (ClC-1, ClC-2, ClC-4, ClC-6, and ClC-7) have been cloned in mammals (for a review, see Ref. [1]), but no amphibian orthologs have been reported so far. ClC-1 is expressed in skeletal muscle where it is involved in the stabilisation of membrane potential. ClC-2 is ubiquitously expressed, slowly activated upon hyperpolarization, cell swelling, and extracellular acidification. Its physiological function(s) remains to be established. In the apical membrane of rat choroid plexus, an inward-rectifying anion conductance with a significant permeability for HCO_3^-, closely resembling the ClC-2 conductance has been identified [101]. Interestingly, a similar anion conductance permeable for HCO_3^- was also described in amphibian choroid plexus [102]. ClC-4, ClC-6, and ClC-7 have broad tissue distributions and are likely to be intracellular. The physiological function(s) of ClC-4 and ClC-6 remain to be elucidated. Disruption of ClC-7 leads to osteopetrosis in mice and human [103].

In addition to ClC-K, ClC-3, and ClC-5, amphibians might possess other ClC family members. Future studies should address this question and elucidate whether ClC channels serve the same functions in amphibian as in mammals.

7. Concluding remarks

Amphibians provide model systems to study transepithelial transports involved in ionic homeostasis and acid–base balance, and cell volume regulation. The fundamental lines defining active sodium reabsorption were established in 1958 by Koefoed-Johnsen and Ussing [14], when the frog skin epithelium was mounted between two chambers and the two-membrane model for Na^+ uptake was first described. Since then, studying the frog skin epithelium provided us with numerous insights into the various transport mechanisms underlying different cell physiological processes. More then two decades later, modern molecular biology tools became available and allowed the molecular identification of pumps, transporters, and channels. It took yet another decade before the first chloride channel was cloned from an amphibian organism: *Xenopus* CFTR. Subsequently, three members of the ClC family of chloride channels have also been identified from *Xenopus*: ClC-K, ClC-3, and ClC-5. However, the functional expression of a cloned channel protein does not necessarily provide an immediate understanding of its physiological function.

The amphibian CFTR was localised in MR cells of the skin epithelium of the toad, and has been proposed to function in the transepithelial chloride transport under control of the β-adrenergic receptor [14].

Xenopus ClC-K is not yet characterised at the electrophysiological level. The recent identification of barttin as a β subunit for ClC-K shed light on the functioning of ClC-K

channels [71]. Future studies aiming at the functional characterisation of ClC-K in *Xenopus* will have to determine whether the function of the amphibian ClC-K also requires the association with an amphibian homologue of barttin.

ClC-3 and ClC-5 appear as intracellular chloride channels in *Xenopus* as in mammals. Functional models are proposed involving both proteins in acidification of intracellular compartments, by providing the electric shunt for electroneutral functioning of V-type ATPases. Future studies ought to examine the possibility that in some cell types (i.e. MR cells in the skin epithelium and intercalated cells in the kidney), these chloride channels might localise at the plasma membrane to function in proton excretion in parallel with plasma membrane proton pumps.

Acknowledgements

It is a pleasure to thank Corinne Cousin and Stéphanie Bogliolo for excellent technical assistance. We also thank T. Jentsch (Hamburg) for providing us with reagents. Cloning of xClC-3 and xClC-5 was done in collaboration with N.K. Wills. Work in our laboratory is funded by the Centre National de Recherche Scientifique (CNRS), the Commissariat à l'Energie Atomique (CEA) and the University of Nice-Sophia Antipolis.

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