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pH and external Ca^{2+} regulation of a small conductance Cl^- channel in kidney distal tubule

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Abstract

A single channel characterization of the Cl^- channels in distal nephron was undertaken using vesicles prepared from plasma membranes of isolated rabbit distal tubules. The presence in this vesicle preparation of ClC-K type Cl^- channels was first established by immunodetection using an antibody raised against ClC-K isoforms. A ClC-K1 based functional characterization was next performed by investigating the pH and external Ca^{2+} regulation of a small conductance Cl^- channel which we identified previously by channel incorporation experiments. Acidification of the *cis* (external) solution from pH 7.4 to 6.5 led to a dose-dependent inhibition of the channel open probability P_O. Similarly, changing the *trans* pH from 7.4 to 6.8 resulted in a 4-fold decrease of the channel P_O with no effect on the channel conductance. Channel activity also appeared to be regulated by *cis* (external) Ca^{2+} concentration, with a dose-dependent increase in channel activity as a function of the *cis* Ca^{2+} concentration. It is concluded on the basis of these results that the small conductance Cl^- channel present in rabbit distal tubules is functionally equivalent to the ClC-K1 channel in the rat. In addition, the present work constitutes the first single channel evidence for a chloride channel regulated by external Ca^{2+}.

Keywords: Cl^- secretion; ClC-K1; ClC-K2; Rabbit; Nephron

1. Introduction

Cl^- channels are known to play a prominent role in a variety of kidney related physiological processes, such as cell volume regulation, urinary acidification, water and solute adsorption, membrane potential stabilization and endosomal acidification [1]. Molecular studies have identified a class of Cl^- selective channels, distinct from the cystic fibrosis transmembrane conductance regulator (CFTR), termed ClC with ClC-K channels exclusively expressed in the kidney [2–4]. Three ClC-K isoforms have been reported, namely hClC-Ka and hClC-Kb in human, rbClC-Ka and rbClC-Kb in rabbit and rClC-K1 and rClC-K2 in the rat [5–8]. The rat rClC-K channels are highly homologous sharing 80% amino acid identity. Similarly, the amino acid sequence of the rbClC-Ka channel is 80–85% homologous to the rat rClC-K1 and rClC-K2 channels and to the human isoforms hClC-Ka and hClC-Kb [8]. Except for the rClC-K1 isoform, none of the ClC-K channels cloned so far has been functionally expressed, thus preventing a characterization of the channel gating and permeation properties. The expression of rClC-K1 channels

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in *Xenopus* oocytes was found, however, to induce outwardly rectifying Cl^- currents that could be inhibited by external acidic pH and by reducing the extracellular calcium concentration [2,9]. Perfusion studies have also indicated that rClC-K1 mediates transepithelial Cl transport in the thin ascending limb of Henle's loop [2] and could play a role in the countercurrent system for urine concentration in the inner medulla [10]. Accumulating evidence suggests that rClC-K1 and rClC-K2 are expressed in distinct regions of the kidney. Whereas the expression of rClC-K1 appears to be most important in the thin ascending limb of Henle's loop (tAL), the presence of rClC-K2 mRNA has been confirmed in the distal convoluted tubule, the connecting tubule and the cortical collecting duct [3]. However, it should be pointed out that the rabbit homologue of rClC-K2 (rbClC-Ka) has been reported as a basolateral Cl^- channel involved in chloride reabsorption in medullary thick limb (MTL) cells [11]. These findings suggest some species-related differences in the localization of the ClC-K channels in various nephron segments. Transcellular Cl^- transport has also been associated with hClC-Kb, the human homologue of rClC-K2. Mutations of the hClC-Kb gene (ClCNKB) were reported to be related to the Bartter's syndrome, an autosomal recessive salt wasting disorder characterized by a reduced NaCl reabsorption in the thick ascending limb (TAL)[12]. In contrast to the kidney-specific ClC-K channels, ClC-2 [13], ClC-3 [14], ClC-4 [15], ClC-5 [16], ClC-6 and ClC-7 [17] are expressed in many tissues. Most attention in this regard has been given to ClC-5 since various mutations of this channel were shown to be associated to Dent's disease and other hereditary syndromes related to kidney stone formation [18]. A prominent role has been attributed to ClC-2 and ClC-3 in cell volume related processes [19,20].

A functional description of ClC-K type Cl^- channel at the single channel level is still lacking. However, recent studies on the rClC-K1 channel have confirmed that the Cl^- currents associated with the expression of rClC-K1 are activated by extracellular Ca^{2+}, a unique feature not shared by other members of the ClC family [2,9,21]. This functional property can thus be used to establish the molecular nature of Cl^- channels measured in kidney cell preparations. In addition, several members of the ClC family are know to be pH sensitive. For instance, a decrease in external pH was found to activate ClC-2 [13], whereas the same pH maneuver led to an inhibition of rClC-K1 [2]. Because of the differential effects observed in response to pH changes, pH-regulation of Cl^- channel activity can also be used as a functional parameter to discriminate among different classes of Cl^- selective channels.

Experiments were thus undertaken to characterize the pH and external Ca^{2+} sensitivity of a small conductance (14 pS in 200 mM $CaCl_2$: 6 pS in 200 mM NaCl) Cl^- channel obtained by fusion of rabbit distal nephron membrane vesicles onto a lipid bilayer. Unlike the Cl^- channels of 9 pS reported in rabbit cortical collecting duct cells (CCD) [22] and at the apical membrane of rabbit distal bright convoluted tubule cells in culture (DCTb) [23], the 14-pS Cl^- channel obtained by incorporation does not require ATP for activation and can be inhibited by the Cl^- channel blocking agents DIDS and NPPB [24], suggesting that it is structurally distinct from CFTR-like channels despite a comparable unitary conductance. Our results show that the distal 14-pS Cl^- channel obtained by incorporation is highly sensitive to variations in both internal (*trans*) and external (*cis*) pH, with a complete inhibition of channel activity in acidic pH conditions ($pH < 6.5$). In addition, we provide, for the first time, evidence that channel activity in this case is regulated by external Ca^2.

2. Materials and methods

2.1. Tubule preparation

Distal and proximal tubules were obtained from rabbit kidneys. The technique of tubule preparation has been described in detail elsewhere [25,26]. Briefly, slices (1–3 mm thick) of rabbit kidney cortex were immersed in a modified Krebs–Henseleit (MKH) solution containing 1 mg collagenase along with 0.5 mg/ml bovine serum albumin (Sigma, St Louis, MO, USA). Following a 20-min digestion period, cold MKH solution was added and the suspension filtered through a tea strainer. The filtrate was centrifuged, washed three times with MKH buffer and suspended in a 45% Percoll in MKH for 20 min. Following centrifugation for 30 min at $28\,000 \times g$,

three bands were clearly formed corresponding, respectively, to distal tubules, glomeruli and proximal tubules. The tubules were collected separately and washed in MKH until free of Percoll. For vesicle preparation, the suspensions of distal tubules were mechanically homogenized (Dounce homogenizer), stirred for 10 min on ice after addition of 12 mM $MgCl_2$ and finally centrifuged at $400 \times g$ for 20 min. The supernatant was collected and centrifuged at $45\,000 \times g$ for 20 min at 4°C. The new sediment was washed twice and suspended in 300 mM mannitol, 15 mM HEPES–Tris at pH 7.4. The resulting membrane preparations lacked glucose-6-phosphatase (endoplasmic reticulum marker), succinate dehydrogenase (mitochondria marker) and glucosamidase (lysosome marker) activities, indicating a low contamination level with membranes from the various internal organelles. However, the membrane preparations used in the present incorporation experiments featured a thiazide-sensitive Na^+ transport and a PTH-dependent Ca^{2+} permeability [25–27], confirming that the vesicles obtained by this procedure originate from plasma membranes of distal nephron cells, including cells from connecting tubules.

2.2. Western blot

Slices of rabbit cortex (200–300 mg) were homogenized using a glass-teflon potter in a 320-mM sucrose solution containing one tablet of Complete Protease Inhibitor Cocktail (Roche Diagnostic) and centrifuged at $2000 \times g$ for 5 min. The surpernatant was then centrifuged at $165\,000 \times g$ at 4°C for 1 h and the pellets were resuspended in 200 μl of the same buffer. The final protein content was estimated by BCA protein assay (Pierce). Proximal and distal fractions were prepared as described above. For each sample, 20 μg of protein were loaded on a SDS–polyacrylamide gel electrophoresis (10%) and proteins were electrotransferred onto a PVDF-membrane (Immobilon-P, Millipore) according to the instructions of the manufacturer. Blots were saturated with 1% BSA in PBS (10 mM sodium phosphate, pH 7.2, 0.9% (w/v) NaCl)–Tween 0.05% and incubated 1 h with a purified polyclonal antibody raised against most ClC-K channels (CLC-K1, CLC-K2, CLC-Ka, CLC-Kb, Clcnka, Clcnkb; Alomone Laboratories) diluted 200-fold. This initial incubation period was followed by an additional 1-h incubation in the presence of horseradish peroxidase-conjugated anti-rabbit IgG or anti-goat IgG (Jackson) with extensive washing steps after antibody incubation. The appropriate bands were finally revealed with the Super Signal (Pierce).

2.3. Single channel recording

2.3.1. Planar bilayer formation

Planar lipid bilayers were formed at room temperature from a 1:1 mixture of 1-palmitoyl-2-oleolphosphatidylcholine (POPC) and 1-palmitoyl-2-oleolphosphatidylethanolamine (POPE) (Avanti Polar Lipids) dispersed in decane at a concentration of 20 mg/ml. The lipid bilayers were painted over a 200-μm hole drilled in a Delrin partition separating two solution-filled chambers of 3 ml (*trans*) and 5 ml (*cis*) respectively. The outline of the aperture was pretreated with a small amount of triglyceride prior to the application of the lipid suspension. Fusion of the vesicles was initiated mechanically by gently touching the bilayer from the *cis* side using a small stainless steel wire (Kerr) of 150 μm diameter, on the tip of which was deposited a small drop of the vesicles containing solution. For right-side-out vesicles, the *cis* chamber corresponds to the extracellular medium. Fusion of membrane vesicles onto a lipid bilayer was systematically carried out in 50 mM NaCl (*trans*)/200 mM $CaCl_2$ (*cis*), pH 7.4 conditions. In experiments where the *cis* Ca^{2+} concentration was varied, the *cis* chamber was perfused after vesicle fusion with 35 ml of a 200 mM NaCl, pH 7.4 solution using a two perfusion pump system (Havard Apparatus 11). Under these conditions, subsequent changes in Ca^{2+} (added from a 1 M $CaCl_2$ stock solution) could be performed in the absence of vesicles in the *cis* compartment. The pH experiments were performed by adjusting the *cis* or *trans* pH value with appropriate amounts of NaOH or HCl. Each amount was tested prior to the single channel experiments by measuring the pH in the *cis* and *trans* chamber using a pH sensitive electrode (Orion Research).

2.3.2. Data acquisition and analysis

Single channel currents were measured using a List EPC7 amplifier. The *trans* chamber was voltage-

clamped relative to the *cis* chamber (ground). Electrical connections were made by using Ag/AgCl electrodes and agar salt bridges (3 M KCl) to minimize liquid junction potentials. Signals were stored on videotapes (SONY, SL-300) and subsequently transferred on hard disk for off-line analysis. Unless specified otherwise, recordings were digitized at a sampling rate of 1 kHz after low-pass filtering at 300 Hz (8-pole Bessel: Frequency Devices 902). The unitary current amplitude and channel open probability, P_O, were estimated from current amplitude histograms or from noise-free Markov signals generated by processing experimental current records using an algorithm based on the Baum–Welch re-estimation formulae [28]. For multi-channel recordings, P_O was calculated assuming that the current levels were distributed according to a binomial statistics. The validity of the binomial distribution was tested by performing a χ^2 analysis based on the procedure described previously [29]. The stationarity of the recordings used for analysis was confirmed according to the criteria described earlier [24].

2.4. Statistical analysis

The data were expressed as means ± S.E.M. Dose–response curves of P_O were fitted to a Hill equation of the form: $P_O = (A1-A2)/(1+[X/X_{1/2}]^N)+A2$, with $X = \{[H], [Ca^{2+}]\}$, N the Hill coefficient and $A1$, $A2$ two adjustable amplitude-related parameters.

3. Results

3.1. Molecular identity of the 14-pS channel

Experiments were first conducted to assay by Western blotting analysis the presence of ClC-K channels in the vesicles formed from distal tubule membranes using an antibody raised against ClC-K channels. A band of the predicted molecular size (83 kDa) was clearly detected in the cortex, distal tubule (DT) and vesicle membrane preparations (Fig. 1). A less prominent band was measured, however, with membranes coming from proximal tubules (PT). These results suggest the presence of ClC-K1 and/or ClCK2-type channels in this vesicle preparation from rabbit distal tubules.

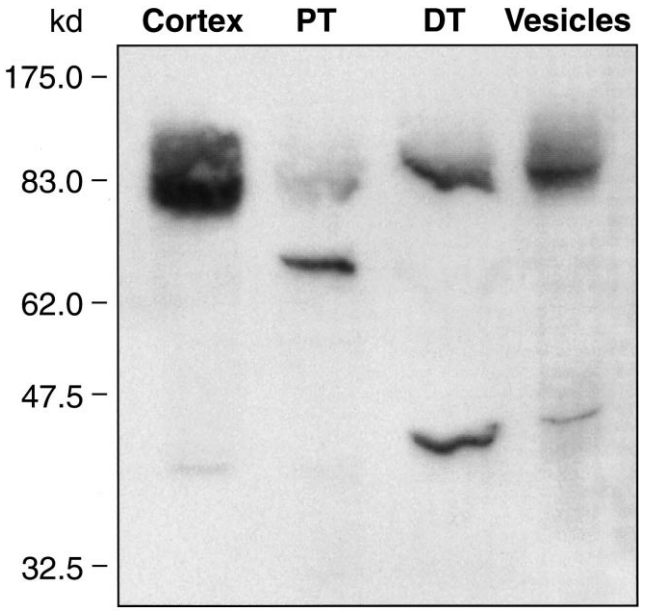

Fig. 1. Immunodetection of ClC-K channels in rabbit distal tubule vesicles. Western blotting was performed using a polyclonal antibody raised against-CLC-K to detect CLC-K1 and CLC-K2 type proteins in rabbit kidney cortex, rabbit proximal tubules (PT), rabbit distal tubules (DT) and vesicles from plasma membranes of rabbit distal tubules, respectively. A band of 83 kDa was clearly apparent in the cortex, distal tubule and vesicle preparations. A band of less important intensity was also detected in proximal tubules. These results confirm the presence of ClC-K channels in the vesicle used for channel incorporation.

3.2. Cl^- channel activity is increased by basic external (cis) pH

Previous experiments where membrane vesicles from purified rabbit distal tubules were fused onto a lipid bilayer have led to the identification of a voltage insensitive Cl^- channel of small conductance (14 pS in 200 mM $CaCl_2$: 6 pS in 200 mM NaCl) [24]. Other anion channels were equally detected, but this channel was the most frequently observed. The effect of external (*cis*) pH was first investigated in a series of incorporation experiments performed in 50 mM NaCl (*trans*)/200 mM $CaCl_2$ (*cis*) conditions. Because the unitary conductance measured under physiological Cl^- concentration (150 mM) was found to be less than 6 pS (data not shown), most experiments were performed using 400 mM Cl^- in the *cis* compartment to insure proper single channel detection. Fig. 2 illustrates single channel current fluctuations recorded at 40 mV (*trans* relative to

cis) for *cis* pH values ranging from 6.8 to 8.0 at a fixed *trans* pH of 7.4. An analysis of the channel current/voltage relationship and pharmacological properties in symmetrical pH conditions (pH 7.4) confirmed that the channel recordings shown in Fig. 2 correspond to the voltage-insensitive 14-pS Cl^- channel described in detail in a previous work [24]. The results of this experiment clearly argue for a regulation of the channel open probability P_O by external (*cis*) pH, with higher P_O values following an alkalinization of the *cis* solution. There was no detectable modification of the channel unitary conductance as confirmed by the current amplitude histograms illustrated in Fig. 2. The pH effect appeared most prominent within the pH range 6.8–7.8, with a 30-fold increase in P_O in response to a 1.0 unit pH increase. Interestingly, decreasing the pH in the *trans* solution from 7.4 to 6.7 resulted in a significant decrease in channel activity despite an alkaline *cis* pH of 8.0. This observation provides evidence for a channel regulation dependent on both the *cis* and *trans* H^+ ion concentrations. The left panel of Fig. 3 summarizes the results of four different experiments performed in 50 mM NaCl (*trans* pH 7.4)/200 mM $CaCl_2$ (*cis*) conditions. The sigmoidal curve was obtained by curve fitting P_O to the Hill equation over the *cis* pH range 6.8–8. The pH for half activation ($pH_{1/2}$) was estimated at 7.44 ± 0.2, with a Hill coefficient N of 1.5 ± 0.5 ($n = 4$), suggesting there were more than one protonation site involved in channel regulation. The mechanism by which external pH affects the channel open probability was next investigated through a Hidden Markov analysis of the current records in Fig. 2. The results of this analysis are presented in Fig. 4A. External pH was found mostly to modulate the channel mean close time with values of 1 s at pH 7.0 compared to 160 ms at pH 8.0, without any significant variation in the channel mean open time with values of 20 ms at pH 7.0 compared to 21 ms at pH 8.0. These observations

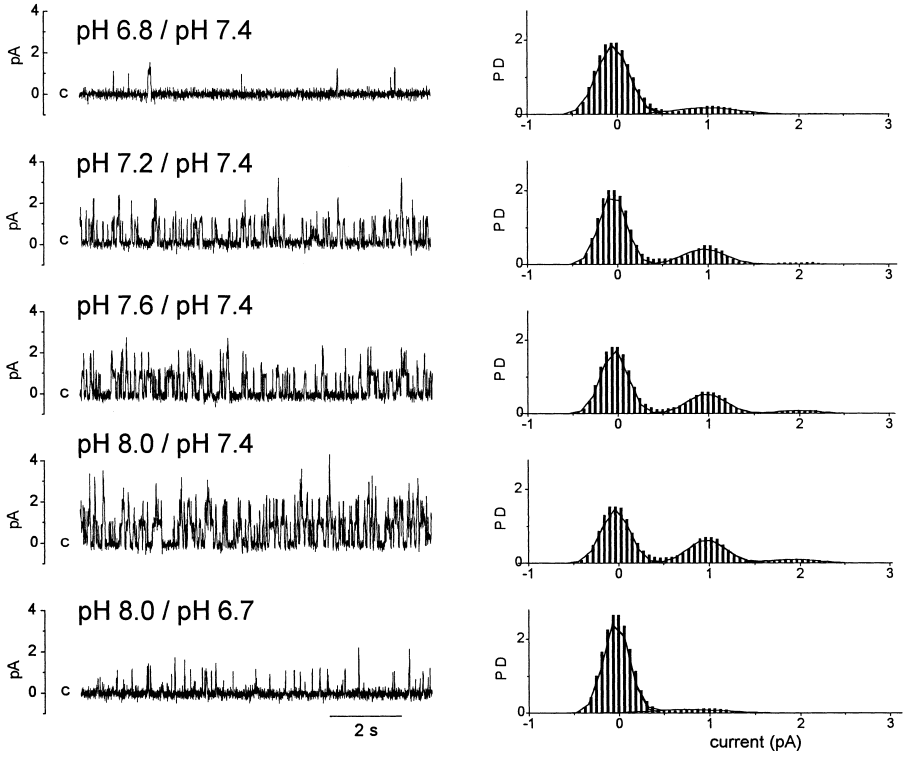

Fig. 2. Effect of external (*cis*) pH on the activity of a 14-pS Cl^--selective channel obtained by fusion of distal tubule membrane vesicles onto a lipid bilayer. Single channel recordings performed at 40 mV (*trans* relative to *cis*) in 200 mM $CaCl_2$ (*cis*)/50 mM NaCl (*trans*) conditions. The associated current amplitude histograms computed from 60 000 events are presented on the right panels. Each histogram was fitted to a summation of Gaussian functions. *cis* pH was varied from 6.8 to 8.0 at a constant *trans* pH value of 7.4, except for the last trace where the *trans* pH was 6.7. Current records were filtered at 300 Hz and sampled at 1 kHz.

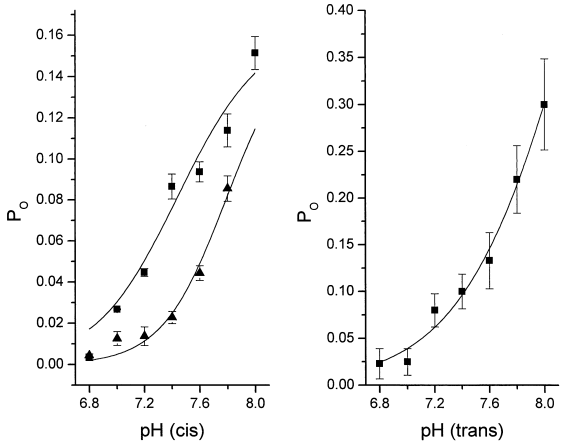

Fig. 3. Dose–response curve of P_O as a function of *cis* pH or *trans* pH. Left panel: P_O variation for *cis* pH values ranging from 6.8 to 8 measured in 200 mM $CaCl_2$ (*cis*)/50 mM NaCl (*trans*) (filled squares) or 200 mM NaCl+20 mM $CaCl_2$ (*cis*)/50 mM NaCl (*trans*) conditions (filled triangles). *trans* pH was maintained to 7.4 throughout. The theoretical curves were computed using a Hill equation with $pH_{1/2}$ 7.44 ± 0.2, and a Hill coefficient N of 1.5 ± 0.5 (n = 4) (filled squares) or a $pH_{1/2}$ 7.78 ± 0. 04 and a Hill coefficient of 1.9 ± 0.3 (n = 3) (filled triangles) respectively. Right panel: P_O variation for *trans* pH values ranging from 6.8 to 8.0 measured in 200 mM $CaCl_2$ (*cis*)/50 mM NaCl (*trans*) conditions (filled squares). Data were fitted to a Hill equation with $pH_{1/2} > 8.4$ and an apparent Hill coefficient of 1.10 ± 0.03 (n = 3).

point towards a stabilization of the channel closed state(s) in acidic pH conditions, with open to close transition rates independent of pH variations.

3.3. Cl^- channel regulation by internal (trans) pH

Fig. 5 shows the effect of the *trans* pH on the activity of the 14-pS Cl^- channel measured at 40 mV in 50 mM NaCl (*trans*)/200 mM $CaCl_2$ (*cis*) conditions. The pH of the *cis* solution was maintained at 7.4 throughout. There was a clear increase in channel activity as the *trans* pH was changed from 6.8 to 8.0. An analysis of the resulting current amplitude histograms confirmed that there was no detectable effect of *trans* pH on the channel unitary conductance (Fig. 5). These results argue for a strong internal pH regulation of the Cl^- channel leading to a significant increase in Cl^- ion permeability over a 1.0 pH unit. Similar results were obtained at a constant *cis* pH of 8.0 (data not shown). The variation in P_O for *trans* pH values ranging from 6.8 to 8.0 is illustrated in the right panel of Fig. 3. Data points from three different experiments were fitted to the Hill equation resulting in $pH_{1/2} > 8.4$ with a Hill coefficient of 1.10 ± 0.03 (n = 3), suggesting the presence of a single protonation site sensitive to *trans* pH. Fig. 4B illustrates the variation in the channel mean open and close times as a function of the *trans* pH value. The alkalization of the *trans* medium appears to decrease the channel mean close time, while increasing the channel mean open time. The latter effect was not observed when the *cis* pH was changed from 6.8 to 8.0 (Fig. 4A), an indication that *cis* and *trans* pH act distinctively on the channel kinetic behavior.

3.4. Effect of external Ca^{2+} on the Cl^- channel activity

It was previously reported that a high *cis* Ca^{2+} concentration was required to insure a significant level of channel activity after fusion of distal membrane vesicles onto a lipid bilayer [24]. In addition, recent studies have provided evidence that rClC-K1 channels are regulated by external Ca^{2+} [9,21]. To follow-up on these observations, the *cis* Ca^{2+} sensitivity of the 14-pS Cl^- channel was investigated in incorporation experiments where the *cis* Ca^{2+} concentration was increased from 5 to 60 mM in symmetrical pH conditions (pH 7.4). Examples of single channel recordings measured at 40 mV (*cis* relative to

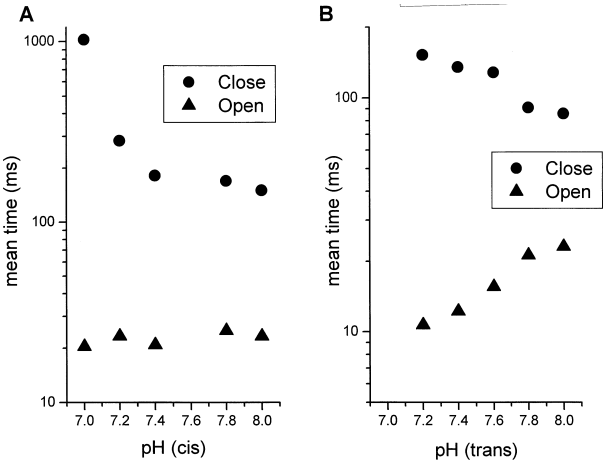

Fig. 4. Effect of *cis* and *trans* pH on the 14-pS channel mean open and close times. Hidden Markov analysis carried on single channel recordings in Figs. 2 and 5. Mean open and close times were estimated from the rates of transition between current levels in multi-channel recordings.

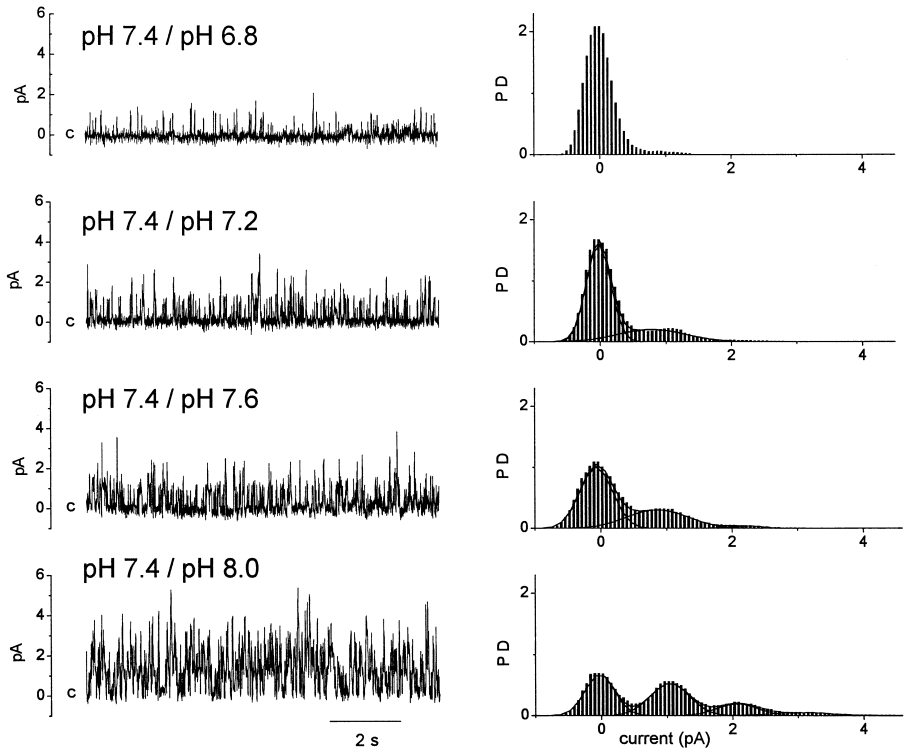

Fig. 5. Effect of intracellular pH (*trans*) on the 14-pS Cl^- activity. Left panels: incorporation experiments performed at 40 mV (*trans* relative to *cis*) in 200 mM $CaCl_2$ (*cis*)/50 mM NaCl (*trans*) conditions. The pH in the *cis* solution was maintained at 7.4 throughout. Current records were filtered at 300 Hz and sampled at 1 kHz. Right panels: associated current amplitude histograms. Each histogram was fitted to a summation of Gaussian functions.

trans) are presented in Fig. 6. There was a significant increase in P_O as the *cis* Ca^{2+} concentration was gradually raised from 5 to 60 mM confirming that the 14 pS obtained by incorporation was sensitive to external Ca^{2+}. The observed effect was not due to an increase in the number of active channels since these experiments were performed following extensive perfusion of the *cis* chamber with a vesicle-free solution. A Hidden Markov analysis of the current records illustrated in Fig. 6 indicated furthermore that the action of external Ca^{2+} on P_O consisted in an increase of the channel mean open time (<2 ms at 5 mM Ca^{2+}, 10 ms at 20 mM Ca^{2+} and 21 ms at 60 mM) coupled to a decrease of the channel mean close time (110 ms at 10 mM Ca^{2+} compared to 55 ms at 60 mM Ca^{2+}). Both mean time values remained, however, constant at Ca^{2+} concentration higher than 60 mM. The effect of the external Ca^{2+} on P_O is summarized in Fig. 7. The Ca^{2+} concentrations for half activation ($Ca^{2+}_{1/2}$) measured in symmetrical 7.4 pH conditions was estimated to 30 ± 2 mM ($n=4$) with a Hill coefficient of 3.0 ± 0.3. The alkalinization of the *cis* medium shifted the Ca^{2+} dose–response curve to the left for a $Ca^{2+}_{1/2}$ equal to 8 ± 3 mM ($n=3$) and a Hill coefficient of 3.0 ± 0.5. The data in Fig. 7 support, therefore, a model whereby the 14-pS Cl^- in distal tubule contains multiple external Ca^{2+} binding sites, the affinity of which increases as the H^+ ion concentration decreases.

The specificity of the divalent cation mediated increase in P_O was next investigated in experiments where Ba^{2+} was added to a *cis* solution containing 40 mM Ca^{2+} (Fig. 8). The addition of 35 mM Ba^{2+} to the Ca^{2+} solution failed to significantly increase P_O, whereas raising the *cis* Ca^{2+} concentration from 40 mM to 60 and 100 mM, respectively, caused a 150% and a 180% increase in channel activity (Fig. 7). This observation argues for a high specificity of the external Ca^{2+} action, while ruling out other mechanisms, such as the electrostatic screening of gating charges or an effect of the *cis* Cl^- concentration on the channel activity.

3.5. Effect of external Ca^{2+} on external pH regulation of the Cl^- channel

To estimate the extent of the channel regulation by external pH at lower Ca^{2+} concentrations, the combined effect of *cis* pH and *cis* Ca^{2+} was investigated in a series of experiments where the *cis* Ca^{2+} concentration was reduced from 200 to 20 mM. We used a minimum *cis* Ca^{2+} concentration of 20 mM to insure a sufficiently large number of single channel openings over a short time period (1 min) for proper P_O determination [24]. Examples of single channel recordings obtained at various *cis* pH values under these conditions are illustrated in Fig. 9. In agreement with the results presented in Fig. 2, there was an important increase in single channel activity as the *cis* solution became more alkaline. The variation in P_O for *cis* pH values ranging from 6.8 to 7.8 is illustrated in the left panel of Fig. 3. Globally, the effect of a 10-fold reduction in the *cis* Ca^{2+} concentration consisted in a rightward shift of 0.35 pH unit in the P_O dose–response curve. In addition, the Hill coefficient increased slightly from 1.5 ± 0.5 ($n = 4$) at 200 mM *cis* Ca^{2+} to 1.9 ± 0.3 ($n = 3$) at 20 mM *cis* Ca^{2+}. These observations argue for a pH regulation of the Cl^- channel activity under normal physiological conditions.

4. Discussion

This paper documents the pH regulation of a 14-pS Cl^- channel recorded after fusion onto a bilayer of plasma membrane vesicles from rabbit distal tubules. Our results support a substantial increase in channel activity following alkalinization of either the *cis* (external) or *trans* (internal) medium within the pH range 6.8–8.0. Globally, pH regulation appeared to be mediated through an increase in P_O, without any significant change in the channel unitary conduc-

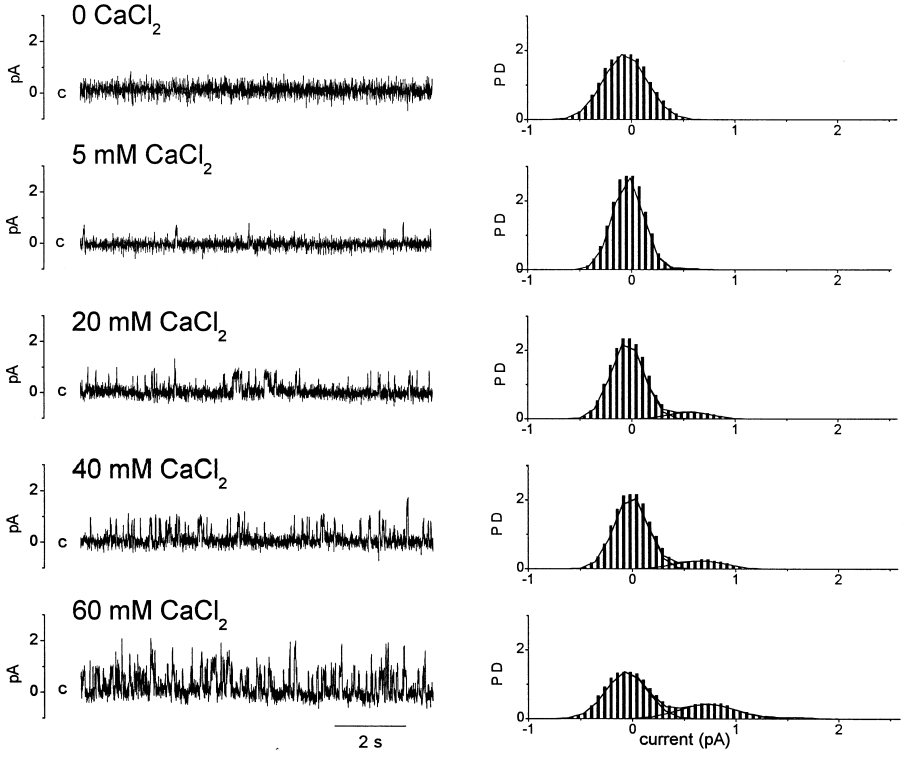

Fig. 6. Regulation of the 14-pS Cl^- channel by external (*cis*) Ca^{2+}. Current fluctuations recorded at 40 mV (*trans* relative to *cis*) in 200 mM NaCl (*cis*)/50 mM NaCl (*trans*) conditions. The pH of the *cis* and *trans* solutions was set to 7.4. Calcium was added directly to the *cis* chamber from a 1-M $CaCl_2$ stock solution. Current records were filtered at 300 Hz and sampled at 1 kHz. Associated current amplitude histograms are presented in the right panels. Note the increase in unitary current amplitude as the $CaCl_2$ concentration is raised from 5 to 60 mM due to an augmentation of the Cl^- ion concentration.

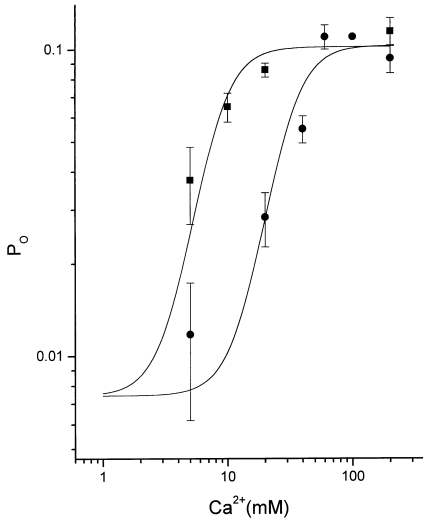

Fig. 7. Dose–response curve of the 14-pS Cl^- channel P_O as a function of the external (*cis*) Ca^{2+} concentration. Filled circles: data points obtained from four different experiments carried in symmetrical pH 7.4 conditions. Sigmoidal curve was calculated using a $Ca^{2+}_{1/2}$ value of 30 ± 2 mM and a Hill coefficient equal to 3.0 ± 0.3. Filled squares: P_O measurements performed in *cis* pH 7.8/*trans* pH 7.4 conditions. Results were fitted to a sigmoidal curve with $Ca^{2+}_{1/2}$ equal to 8 ± 3 mM and a Hill coefficient of 3.0 ± 0.5 ($n = 3$).

tance. We also observed that channel activity was a function of the *cis* Ca^{2+} concentration, with an increase in open channel probability at higher *cis* Ca^{2+} concentration values. These observations, together with results from immunodetection experiments with an anti-ClC-K-antibody, suggest that the 14-pS Cl^- channel in rabbit distal nephron could correspond to a rClC-K1 type channel.

4.1. Effect of pH on Cl^- channels

The results in Figs. 2 and 5 show that the 14-pS Cl^- channel from distal plasma membrane vesicles is regulated by both *cis* and *trans* pH. For instance, the data presented in Fig. 3 indicate that an alkalinization of the external (*cis*) medium from pH 7.2 to 7.4 is associated with a 200% increase in channel activity. Similarly, changing the *trans* (internal) pH from 7.2 to 7.4 caused a 160% augmentation in the channel open probability (Fig. 3B). Our results support, therefore, a mechanism whereby intra-or extracellular physiological pH changes result in an enhanced Cl^- permeability in distal tubule. This effect was entirely mediated by a modulation of P_O, suggesting that the titration process most likely affects sites essential to channel gating rather than residues responsible for the channel permeation properties. A $pH_{1/2}$ of 7.4 for the P_O dose–response curve as a function of *cis* pH would be within the pH range expected for the ionization of histidine residues.

A regulation of Cl^- channel activity by external pH has been documented in several cellular preparations. In contrast to the inhibitory effect of acidic *cis* and *trans* pH illustrated in Figs. 2 and 5, low pH values have been reported to activate several key Cl^- channels. For instance, an enhancement of the ClC-1 inward steady-state current has been measured following a decrease in external pH [30]. Similarly, the ClC-2 channel activity was increased by acidic external pH through a shift of the channel voltage dependency towards more positive potentials [13]. These observations confirm that the 14-pS Cl^- channel described in the present work is distinct from ClC-1 and ClC-2 type Cl^- channels.

A regulation by external pH was also reported for an inwardly-rectifying Cl^- channel in rat choroid plexus [31] and for the amphibian and human ClC-5 channels [32] with channel inhibition observed at acidic pH. These findings are in agreement with the external (*cis*) pH modulation illustrated in Fig. 2. However, ClC-5 appeared to be insensitive to channel blocking agents, such as DIDS (1 mM) and DPC (1 mM) [24,32], indicating that ClC-5 and the present Cl^- channel are otherwise different [24]. A 9-pS cAMP-dependent Cl^- channel present in the mouse thick ascending limb was equally reported to be inhibited at low internal pH [33]. As in the present case, channel inhibition caused by high internal H^+ concentration was not mediated by a decrease in single channel conductance, but was consequent to a decrease in the channel open probability P_O. Our results point, however, toward a greater pH sensitivity, as P_O increases more steeply between pH 7.4 and 8.0 for the 14-pS channel (Fig. 3, right panel) than for the Cl^- channel in the mouse thick ascending limb [33]. These observations coupled to the fact that both channels share similar permeation and pharmacological properties, suggest some level of homology at the molecular level [24,33,34]. However, in contrast to the Cl^- channel of the mouse thick ascending limb, a sustained channel activity could be

recorded in incorporation experiments in the absence of ATP, indicating that the two channels belong to different Cl^- channel families. Finally, the results presented in the left panel of Fig. 3, are compatible with the external pH dependency reported for the kidney specific ClC-K1 channel expressed in *Xenopus* oocytes [2,9]. Interestingly, ClC-K1 is also known to be voltage-independent, activated by external Ca^{2+} and sensitive to DIDS with a K_i within the mM concentration range [6]. These features correspond to our published and current description of the 14-pS Cl^- channel [24] and suggest ClC-K1 as a valuable molecular candidate.

4.2. Regulation by external (cis) Ca^{2+} concentration

The results presented in Figs. 6 and 7 provided the first single channel evidence for a chloride-selective channel regulated by external Ca^{2+}. The effect appeared to be independent of the fusion process, since the number of channels recorded following channel incorporation remained constant. Indeed, experiments were performed such that the *cis* (external) chamber was systematically perfused with 35 ml of a 200 mM NaCl solution to remove extraneous vesicles. Hence, the open channel probability could be directly measured without making any assumption regarding the number of active channels (see Section 2). In contrast to the well-documented calcium-activated Cl^- channels, the Ca^{2+} dependency in this case involves external Ca^{2+} and occurs within the mM range. Interestingly, the addition of external $BaCl_2$ (up to 55 mM) (Fig. 8) to a solution containing 40 mM $CaCl_2$ failed to mimic the effect caused by an increase in *cis* Ca^{2+} from 40 to 60 mM (Fig. 6) or 100 mM (data not shown). This observation suggests a specificity of the Ca^{2+} action, ruling out other mechanisms, such as electrostatic charge screening or channel activation due to an increased Cl^- concentration in the *cis* solution [1]. The latter conclusion is also supported by the fact that raising the *cis* NaCl concentration from 200 to 440 mM in 20 mM

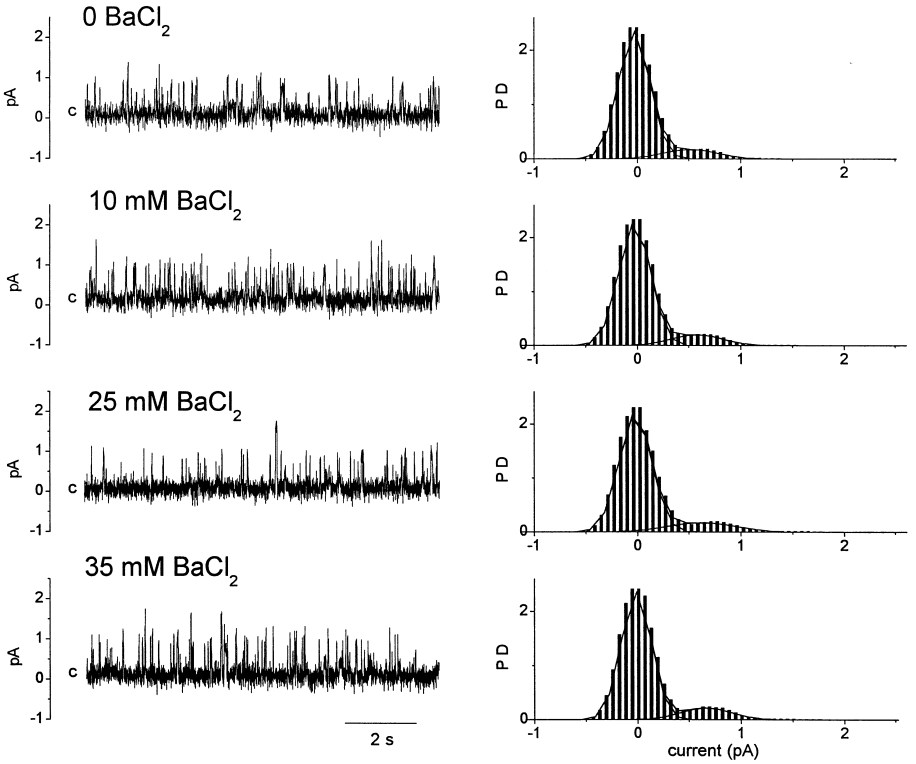

Fig. 8. Specificity of the external Ca^{2+} regulatory effect. Incorporation experiments were performed at 40 mV in 160 mM NaCl+40 mM $CaCl_2$ (*cis*)/50 mM NaCl (*trans*) conditions. The pH of the *cis* and *trans* solutions was set to 7.4. Ba^{2+} was added directly to the *cis* chamber from a 1-M $BaCl_2$ solution. Filtering and sampling are as described in previous figures.

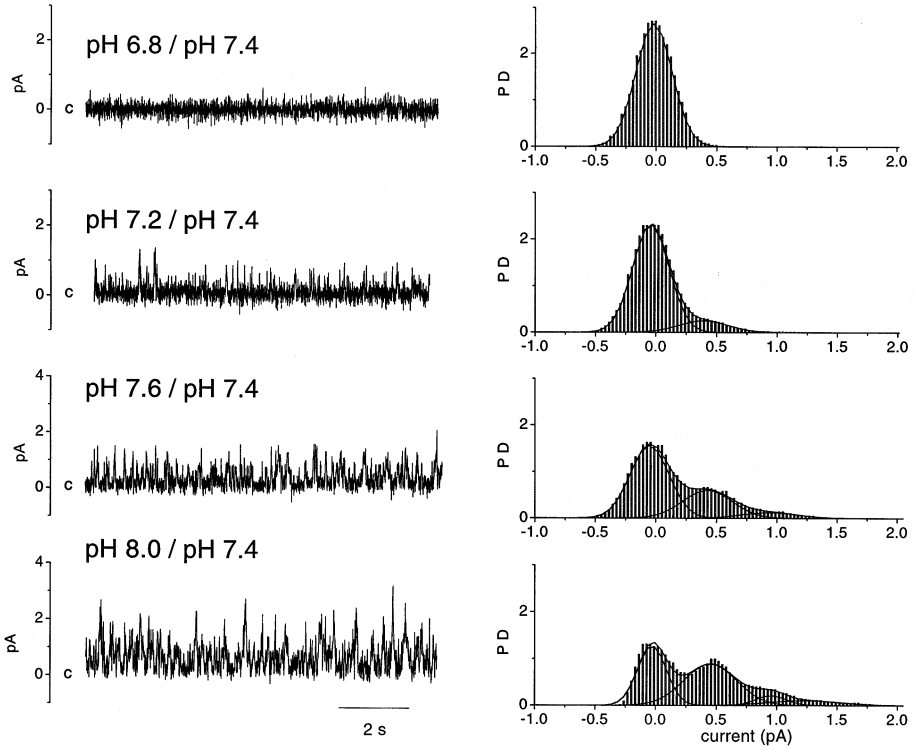

Fig. 9. Effect of external Ca^{2+} on the external (*cis*) pH regulation of the 14-pS Cl^- channel. Left panels: current fluctuations recorded at +40 mV (*trans* relative to *cis*) in 200 mM NaCl+20 mM $CaCl_2$ (*cis*)/50 mM NaCl (*trans*) conditions. Filtering and sampling as described previously. Right panels: associated current amplitude histograms. Current amplitude was estimated at 0.48 pA for a unitary conductance of less than 6 pS.

$CaCl_2$ conditions did not result in a greater number of channel openings (data not shown). The external Cl^- concentration did not appear, therefore, as an important determinant to of the 14-pS channel activity for Cl^- concentrations higher than 200 mM. This is distinct from the Cl^- channels in basolateral membranes of medullary TAL cells where a $[Cl]_{1/2}$ for channel gating by extracellular Cl^- of 175 mM was reported [11]. The single channel data presented in this work confirm that the 14-pS Cl^- channel activity requires the presence of external Ca^{2+} as no channel activity could be recorded under zero external Ca^{2+}. However, single channel events were clearly detectable in the presence of 5 mM *cis* Ca^{2+} (see Fig. 6) at *cis* pH of 7.4 and 7.8 despite a relatively low open probability (0.01). It is clear from our results that measurements at the single channel level of this Cl^- channel under physiological external Ca^{2+} conditions require an alkaline external pH (pH > 8.0) for optimum detection. Our results support, therefore, a model where alkaline external pH conditions promote channel activity through an external Ca^{2+}-dependent process. A Ca^{2+}-dependent regulation was not observed in response to variations in *trans* Ca^{2+} concentration since experiments carried out either in 50 mM NMDG-Cl (*trans*)/200 mM $CaCl_2$ (*cis*) or 50 mM $CaCl_2$ (*trans*)/200 mM (*cis*) $CaCl_2$ conditions led to the same open channel probability [24]. A regulation by external Ca^{2+} was reported for the rat kidney rClC-K1 channel expressed in *Xenopus* oocytes [2,9,35] and for ClC-K1 channels present in marginal cells of the stria vascularis [21]. Double electrode voltage clamp and whole cell recordings have confirmed, in both cases, that the removal of external Ca^{2+} results in a 60–70% decrease of the overall chloride current [2,21]. In contrast, a 4-fold increase of the Cl^- current amplitude mediated by rClC-K1 expressed in *Xenopus* oocytes could be initiated following an elevation of the extracellular Ca^{2+} concentration from 1 to 5 mM [9]. The latter effect appeared to be Ca^{2+}-specific since the addition of 5 mM magnesium or barium failed to initiate an

increase of the rClC-K1 mediated chloride currents under identical recording conditions [9]. It was suggested, on the basis of these observations, that the regulation by Ca^{2+} of ClC-K1 involves an extracellular domain of the protein [2]. These results are in agreement with the findings presented in Figs. 6 and 8 and suggest that the channel obtained by incorporation possesses features compatible with a ClC-K1 type channel. This conclusion is further supported by the immunodetection of ClC-K channels in the vesicle preparations used for channel incorporation (Fig. 1). These observations, together with the fact that rClC-K1 and 14-pS channels are both voltage insensitive, inhibited by external acid pH and activated by external Ca^{2+} while sharing the same pharmacological properties, argue for a strong structural homology in this case. This would imply, however, that the ClC-K1-like channel in rabbit is not exclusively localized in the tAL as observed for rClC-K1, but is also present in the cortical portion of the kidney. Patch-clamp experiments remain needed to confirm if the single channel features of rClC-K1 correspond to that associated with the current 14-pS channel.

5. Conclusion

In conclusion, our results provided evidence for a Cl^- channel in rabbit distal tubule that is highly sensitive to variations in both internal and external pH within a physiological pH range (6.8–7.4). A regulation by external Ca^{2+} was also observed suggesting a potential interaction between luminal Ca^{2+} and Cl^- ion transport in distal tubule. Finally, the extracellular pH dependency and the sensitivity to external Ca^{2+} point toward a channel with ClC-K1 properties.

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