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Research Article

Lack of Na^+,K^+-ATPase expression in intercalated cells may be compensated by Na^+-ATPase: A study on MDCK – C11 cells

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Abstract. The lack of Na^+,K^+-ATPase expression in intercalated cells (IC) is an intriguing condition due to its fundamental role in cellular homeostasis. In order to better understand this question we compared the activities of Na^+,K^+-ATPase and Na^+-ATPase in two MDCK cell clones: the C11, with IC characteristics, and the C7, with principal cells (PC) characteristics. The Na^+,K^+-ATPase activity found in C11 cells is far lower than in C7 cells and the expression of its β-subunit is similar in both cells. On the other hand, a subset of C11 without α-subunit expression has been found. In C11 cells the Na^+-ATPase activity is higher than that of the Na^+,K^+-ATPase, and it is increased by medium alkalinization, suggesting that it could account for the cellular Na^+-homeostasis. Although further studies are necessary for a better understanding of these findings, the presence of Na^+-ATPase may explain the adequate survival of cells that lack Na^+,K^+-ATPase.

Keywords. Na^+,K^+-ATPase, Na^+-ATPase, MDCK-C11, MDCK-C7, intercalated cells.

Introduction

The Na^+,K^+-ATPase plays an essential role in renal transporting epithelia, generating a transmembrane ionic gradient that drives secondary active transport of different filtered solutes. Although this enzyme has been localized in the basolateral membrane of the majority of cells along the mammalian nephron, several authors could not clearly demonstrate its presence in intercalated cells (IC) of distal tubule and collecting duct [1–4], and even those who were successful agreed that its expression was very low [5, 6]. Due to its fundamental role in the physiological cellular homeostasis, the lack of expression of Na^+,K^+-ATPase in IC is an intriguing condition. On the other hand, the furosemide inhibitable, ouabain insensitive Na^+-ATPase [7, 8] has been detected in proximal tubules and in MDCK cells [9, 10], being modulated by hormones and autacoids such as angiotensin, and adenosine [11–13]. This enzyme could be the alternate molecular mechanism responsible for adequate survival of these cells in the absence of the classical Na^+,K^+-ATPase sensitive to ouabain. Thus, the objective of the present study was to compare the activities of both sodium ATPases in two MDCK cells clones: C11, with IC characteristics, and C7, with principal cells (PC) characteristics.

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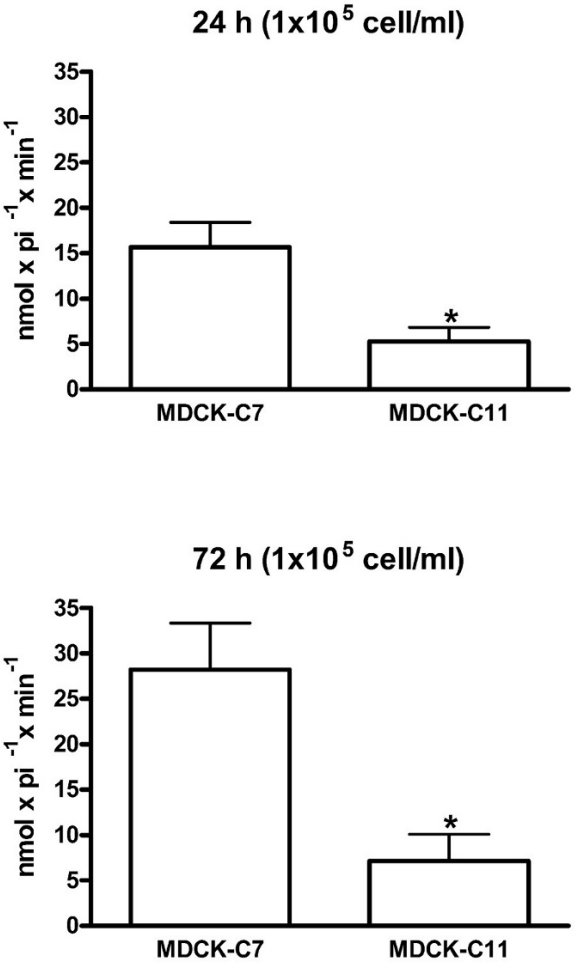


Figure 1. Na^+,K^+-ATPase activities of clones MDCK-C11 and MDCK-C7. The cells were plated at low density (1 x 10^5 cells/ml) in six-well plates and grown for 24 or 72 h. The cells were harvested and the activity of Na^+,K^+-ATPase was measured as stated in Material and Methods. * Difference between means statistically significant ($p<0.05$), $n = 6$.

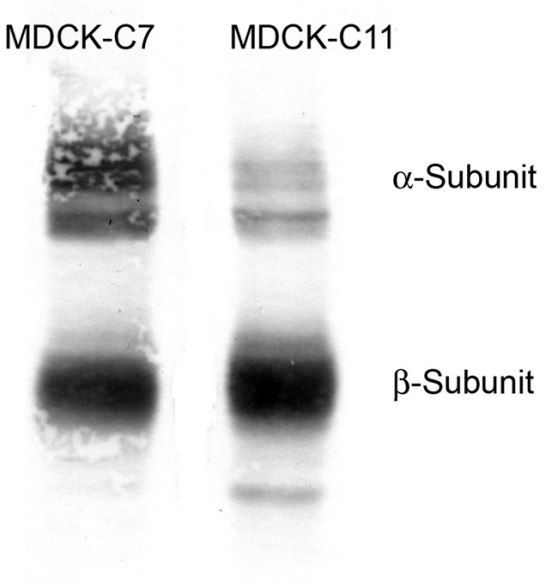


Figure 2. Expression of α1 and β1 subunits of Na^+,K^+-ATPase in MDCK-C11 and MDCK-C7 cells. A representative immunoblot for α1 and β1 subunits of Na^+,K^+-ATPase in cellular extracts prepared from MDCK C7 and C11 cells. Blots of whole cell extracts were probed with specific antibodies to α1 and β1 subunits of Na^+,K^+-ATPase, as described in Material and Methods. Representative blotting obtained from three distinct experiments.

Materials and methods

Cells and Culture Conditions. The epithelial cell lines MDCK-C7 and MDCK-C11 were grown in Dulbecco's Modified Eagle Medium-DMEM (GIBCO, USA) with penicillin and streptomycin (GIBCO, USA) and supplemented with 10% fetal bovine serum (GIBCO, USA) in disposable plastic bottles, at 37°C, until reaching confluence. For each experiment, the cells were seeded in six-well plates at a concentration of 2 x 10^5 cells/ml or in 24-well plates at a concentration of 5 x 10^4 cells/ml.
In some experiments the cells were plated in media adjusted to different pH with NaOH or HCl.

Measurement of ATPase activity. Cells seeded in six-well plates (Corning-Costar Corp, USA) at 2 x 10^5 cells/ml and grown for 24 h or 72 h were solubilized in a solution of deoxycholic acid 0.1%, EDTA 1 mM, Hepes-Tris 20 mM and sucrose 250 mM. All the reagents were obtained from SIGMA, USA. The ATPase activity was measured according to the method previously described [9–13]. The reaction was started by the addition of the homogenates to a final protein concentration of 0.1–0.3 mg/ml, and stopped after 10 minutes by the addition of charcoal activated by HCl (0.1 N). The [^{32}P]Pi released was measured in an aliquot of the supernatant obtained after centrifugation of the charcoal suspension for five minutes at 1,500 *g*. Spontaneous hydrolysis of [^{32}P]ATP was measured simultaneously in tubes with no protein added. The Na^+,K^+-ATPase activity was calculated from the difference between the [^{32}P]Pi released in the absence and in the presence of 1 mM ouabain. The Na^+-ATPase activity was calculated from the difference between the [^{32}P]Pi released in the absence and in the presence of 2 mM furosemide, both in the presence of 1 mM ouabain. Protein concentrations were determined by the Folin phenol method using bovine serum albumin as a standard.

Western Blot Analysis. The only isoforms of Na^+,K^+-ATPase found in the kidneys are the α1 and β1 [14]. Moreover, it has been shown that the parental MDCK cells only express the alpha 1 isoform [15]. Therefore, the expression of α1 and β1 subunits of Na^+,K^+-

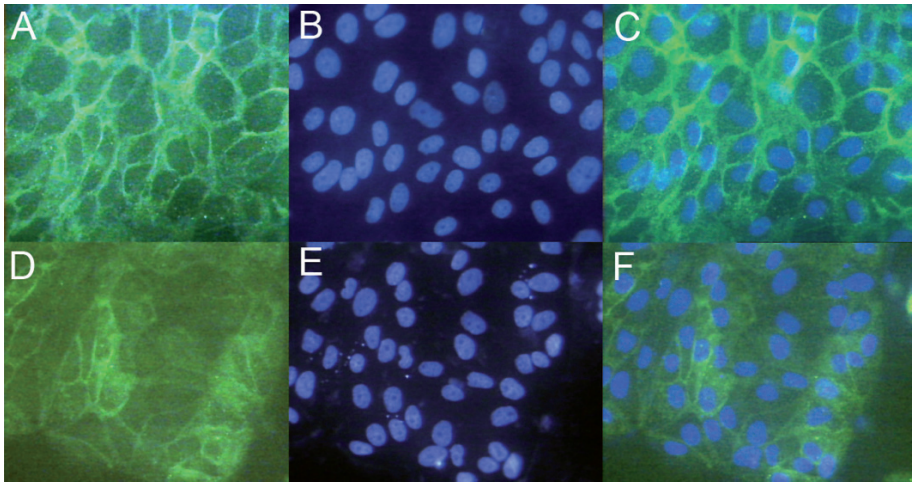


Figure 3. Expression of α1 subunit of Na^+,K^+-ATPase in clones C7 and C11, visualized by immunofluorescence. Cells were grown on coverslips and incubated with an antibody to the α1 subunit of Na^+,K^+-ATPase, as described in Material and Methods. Upper panels: MDCK-C7 cells; Lower panels: MDCK-C11 cells. Left panels: cells labeled with antibody against the α1 subunit of Na^+,K^+-ATPase. Middle panels: the same cells labeled with the nuclear fluorescent dye Hoechst 33258; Right panels: combined images. The figures are representative of three independent experiments.

ATPase in MDCK-C7 and C11 cells was assessed by immunoblotting using specific antibodies (Santa Cruz Biotechnology, USA). After the removal of the culture media, confluent cells were washed three times with PBS (pH 7.4) at room temperature, scraped, and centrifuged at 8,000 *g* for 90 s.
Whole cell extracts were prepared by diluting the cell pellets directly in sample buffer, consisting in 1.5% sodium dodecyl sulfate (SDS), 10 mM Tris[hydroxymethyl]aminomethane (Tris-Cl) pH 6.8, 0.6% dithio-L-threitol (DTT), 0.5% β-mercaptoethanol, and 6% glycerol. The proteins (20 μg) were immediately submitted to 7.5% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA). The membranes were blocked with Western Breeze blocking solution (Invitrogen, USA) and incubated with specific antibodies to α1 and β1 subunits of Na^+,K^+-ATPase (Santa Cruz Biotechnology, USA). The phosphatase alkaline Western Breeze Kit (Invitrogen, USA) was used to visualize the bands in the membranes.

Immunofluorescence. Cells grown on coverslips in 24-well plates for 24 h were fixed and permeabilized with acetone-methanol. After washing, the cells were incubated with 5% bovine serum albumin (BSA, INVITROGEN, USA) in PBS for 30 minutes, washed and incubated for 2 h with a mouse monoclonal antibody to α1 subunit of Na^+,K^+-ATPase (Santa Cruz Biotechnology, USA). After washing, the cells were incubated with ALEXA 488 antimouse secondary antibody (Molecular Probes, Invitrogen, USA) for 30 min. The cells were then washed and incubated with a goat polyclonal antibody to β1 subunit of Na^+,K^+-ATPase (Santa Cruz Biotechnology, USA) for 2 h, washed and incubated for 30 min with anti-goat ALEXA 546 secondary antibody (Molecular Probes, Invitrogen, USA). After washing, the cells were mounted on glass slides with an antifading kit (Molecular Probes, Invitrogen, USA) and the fluorescence was visualized under a fluorescence microscope (Axiovert 100, Zeiss, Germany). In some experiments, cells were also labeled with peanut agglutinin (PNA), a marker of β-intercalated cells, or Hoechst 33258, for nuclear staining.

Statistical Analysis. Each experiment was repeated from three to five times. Data are expressed as means ± standard deviation of the means and were analyzed using Students t-test for comparison of the differences, except for Figure 7, where One-Way ANOVA with Bonferroni's post-test was used. Values of $p < 0.05$ were considered statistically significant.

Results

Figure 1 shows the Na^+,K^+-ATPase activities in MDCK-C11 and MDCK-C7 cells after 24 h and 72 h of growth. The Na^+,K^+-ATPase activity increase in MDCK-C7 cells was time dependent during the period of cell culture. However, independent of time, MDCK-C11 cells have significantly lower Na^+,K^+-ATPase activity when compared with MDCK-C7 cells. In order to verify if this reduced activity could be due to a diminished expression of the α-subunit of Na^+,K^+-ATPase in clone C11, an immunoblotting was performed for α and β subunits. Figure 2 shows that, although the two clones have approximately the same amount of β-subunit, MDCK-C11 cells possess much less α-subunit than MDCK C7 cells. This result suggests that the reduced activity of Na^+,K^+-ATPase in C11 cells is either due to a homogeneous reduced expression of the α-subunit in the population or that, on the other hand, some cells in

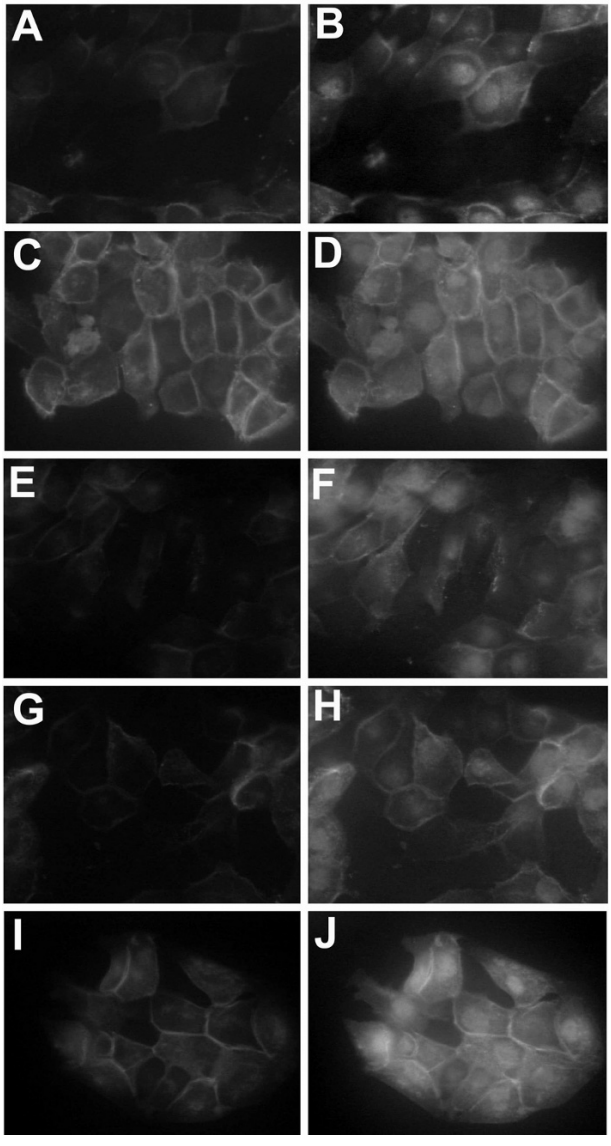


Figure 4. Expression of α1 and β1 subunits of Na^+,K^+-ATPase in MDCK-C11 cells, visualized by immunofluorescence. Cells were grown on coverslips and incubated sequentially with antibodies to the α1 and β1 subunits of Na^+,K^+-ATPase, as described in Material and Methods. Left panels: cells labeled with antibody to the α1-subunit of Na^+,K^+-ATPase. Right panels: the same cells labeled with antibody to the β1-subunit of Na^+,K^+-ATPase. The figures are representative of three independent experiments.

clone C11 express very low amounts of the α-subunit of Na^+,K^+-ATPase. To further study this issue, the expression of the α-subunit of Na^+,K^+- ATPase in both clones was observed by fluorescence microscopy. As can be seen in Figure 3, the α-subunit is present at cell membranes of almost all MDCK-C7 cells (Figure 3 A–C). However, although some MDCK-C11 cells considerably express the α-subunit of Na^+,K^+-ATPase, the majority of the cells have very low expression and there are some cells in clone C11 that virtually do not express this subunit (Figure 3 D–F).

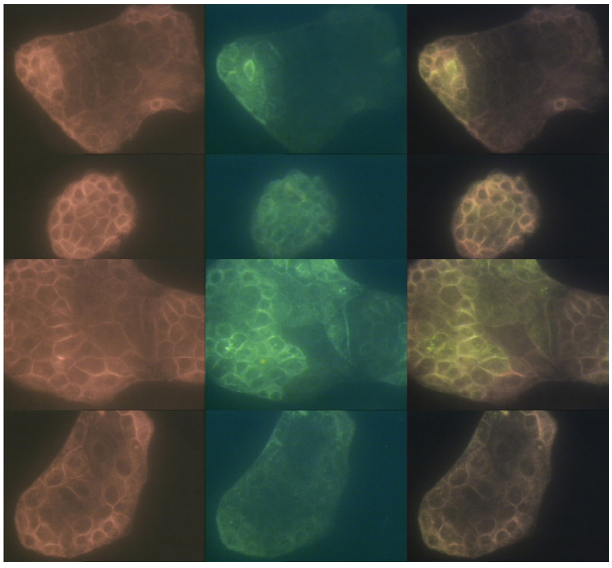
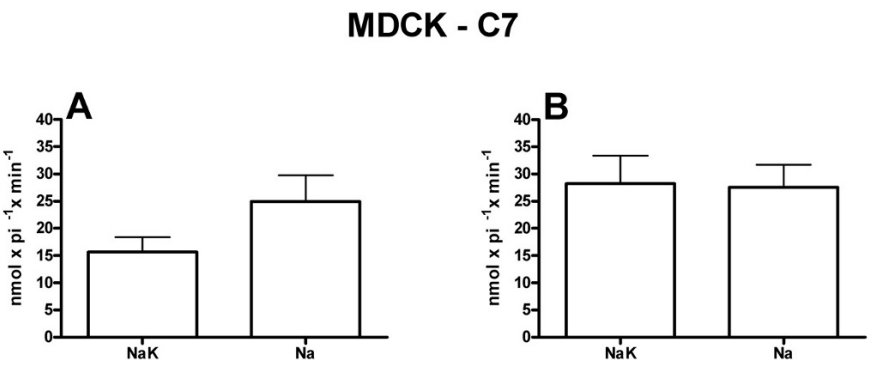


Figure 5. Co-expression of α1 subunit of Na^+,K^+-ATPase and peanut agglutinin (PNA) in C11 cells, visualized by immunofluorescence. Cells were grown on coverslips and incubated with an antibody to the α1 subunit of Na^+,K^+-ATPase and PNA, as described in Material and Methods. Left panels: cells labeled with antibody to the α1- subunit of Na^+,K^+-ATPase. Middle panels: the same cells labeled with PNA; Right panels: combined images. The figures are representative of three independent experiments.

These results confirm that the reduced activity of Na^+,K^+-ATPase in C11 cells is due to reduced expression of the α-subunit. However, since the β-subunit was almost equally expressed in the two cell lines, as seen by immunoblotting, the expression of α and β subunits in MDCK-C11 was also observed by immunofluorescence. Figure 4 shows photographs of C11 cells labeled with anti-α (left panel) and anti-β (right panel) antibodies. Actually we see that there are several cells in clone MDCK-C11 that virtually do not express the α-subunit, although they express the β-subunit.

It has been shown that the majority of cells in clone C11 have characteristics of type β-intercalated cells, although the cells are not homogenous, as expected for a cloned cell line [16]. Moreover, it is known that β-intercalated cells may differentiate to type α-intercalated cells in culture [17–20]. Therefore, to evaluate whether the expression of the α-subunit of Na^+,K^+-ATPase in C11 cells is restricted to type α or type β intercalated cells, the cells were labeled for the α-subunit of Na^+,K^+-ATPase and PNA, a marker of β-intercalated cells. As can be seen in Figure 5, the majority of C11 cells expressing the α-subunit of Na^+,K^+-ATPase was also labeled with PNA, strongly suggesting that type β-intercalated cells are the ones expressing the α-subunit of Na^+,K^+-ATPase.

As Na^+,K^+-ATPase is thought to be fundamental for the maintenance of low intracellular Na^+ concentration, the



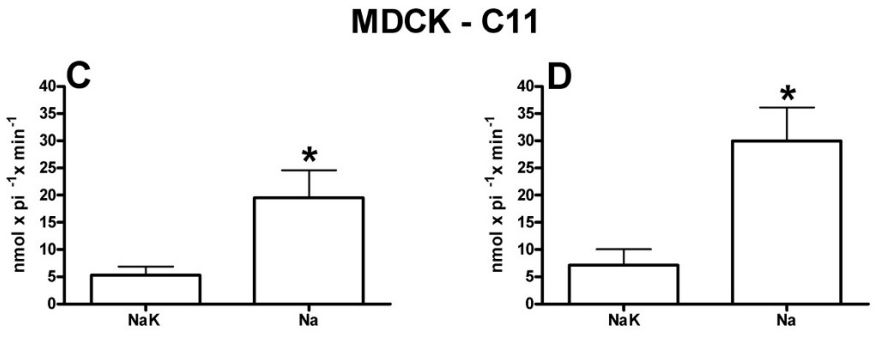


Figure 6. Comparison of Na^+,K^+-ATPase and Na^+-ATPase activities in MDCK-C7 and MDCK-C11 cells. Cells (2×10^5 cells/ml) were plated in six-well plates and incubated for 24 or 72 h. The activities of Na^+,K^+-ATPase and Na^+-ATPase were measured as stated in Material and Methods. A,C – 24 h of incubation; B,D - 72 h of incubation. * Statistically different in relation to the control ($p<0.05$), n = 6.

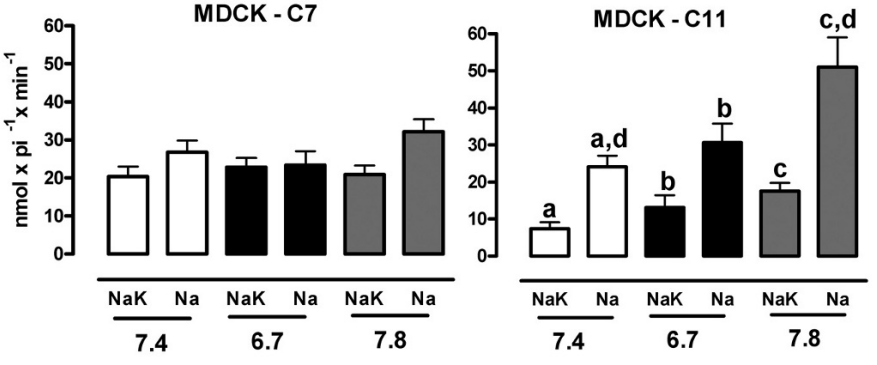


Figure 7. Variation of Na^+,K^+-ATPase and Na^+- ATPase activities in MDCK-C7 and MDCK-C11 cells with pH. The cells were grown for 24 h in media at pH = 6.7 (acidic); 7.4 (normal) and 7.8 (alkaline) and the activities of Na^+,K^+-ATPase and Na^+-ATPase were measured as stated in Material and Methods. Same letters: statistically different between each other ($p<0.05$), n = 6.

absence of this protein could result in loss of viability. However, there is no evidence that this occurs, because the viability of a culture of MDCK-C11 cells is always $\geq$ 95 %, similar to that of MDCK-C7 (data not shown). A possible explanation is that the expression of the second Na^+-pump [7–10] could be responsible for Na^+ handling in these cells. Therefore, we tested whether MDCK-C11 cells posses Na^+-ATPase activity. Figure 6 shows that both Na^+-ATPase and Na^+,K^+-ATPase activities in MDCK-C7 cells are similar, while the activity of Na^+-ATPase in C11 cells is about four- times greater than that of Na^+,K^+-ATPase, suggesting that this protein might be mainly responsible for intracellular Na^+ handling in MDCK-C11 cells.

Intercalated cells are involved in acid-base balance. While type α-intercalated cells are involved in H^+ secretion and HCO_3^- reabsorption, type β cells are thought to secrete HCO_3^- and reabsorb H^+. In all other renal epithelial cells the transport of ion and molecules is secondary to the Na^+ gradient originating from the basolateral Na^+,K^+-ATPase. Therefore, it may be reasonable to hypothesize that the Na^+-ATPase is the Na^+ transport mechanism present in intercalated cells, since the expression of Na^+,K^+-ATPase in such cells is very low or even non-existent [1–6]. To evaluate the significance of Na^+-ATPase in acid-base regulation in intercalated cells, the clones C7 and C11 were exposed to acidic or alkaline pH and the activities of both

Na^+,K^+-ATPase and Na^+-ATPase were measured. Indeed, as can be seen in Figure 7, the activity of Na^+-ATPase was greatly increased at alkaline pH in clone C11, but no significant alteration was observed in the activity of Na^+,K^+-ATPase. In no situation did MDCK-C7 present any significant alteration.

Discussion

The maintenance of a low intracellular Na^+ concentration against its gradient has fundamental importance to a cell, and this feature is due to the presence of Na^+,K^+-ATPase in the majority of the cells. For a long time the very low expression or even absence of Na^+,K^+-ATPase in intercalated cells of collecting duct has intrigued researchers, and several of them have made intensive, although unsuccessful, efforts to show its presence in these cells [1–4]. Even those authors able to demonstrate the expression of Na^+,K^+-ATPase in IC cells agreed that this expression was very low [5, 6]. All those studies were performed in renal tissue or in isolated tubules, which might complicate the visualization of this protein. In the present work, we have studied a previously cloned renal cell line that retains characteristics of IC [16]. Using this approach, we have shown that the Na^+,K^+-ATPase activity of MDCK-C11 cells (which retain characteristics of intercalated cells) is far lower than that of MDCK-C7 cells (which retain characteristics of principal cells). We have also shown that, although the expression of the β-subunit of Na^+,K^+-ATPase was similar in clones C11 and C7, there is a subset of MDCK-C11 cells that virtually does not express the α-subunit. This subset seems to be of type α or non-α non-β intercalated cells, because cells labeling for α-subunit of Na^+,K^+-ATPase are also labeled for PNA, a marker of type β-intercalated cells. This result agrees with literature that shows low or no expression of Na^+,K^+-ATPase in IC, for in a normal kidney the type α intercalated cells are dominant. On the other hand, we have also observed that the activity of the furosemide inhibitable, ouabain insensitive Na^+-ATPase was much higher than that of Na^+,K^+-ATPase, supporting the hypothesis that the ouabain-insensitive Na^+-ATPase could account for the Na^+-homeostasis in intercalated cells. Moreover, the alkalinization of the medium led to a great increase in Na^+-ATPase activity only in MDCK-C11 cells, corroborating the hypothesis that this second pump is of crucial importance for the homeostasis of intercalated cells. Gekle et al. [16] showed that under chronic alkaline stress MDCK-C7 cells were unable to maintain their intracellular pH within normal limits, suffering genetic and morphologic alteration, while MDCK-C11 cells were able to regulate their intracellular pH. The great increase in Na^+-ATPase activity in MDCK-C11 cells and no alteration in its activity in MDCK-C7 cells suggest that this protein may be involved in pH regulation in intercalated cells.

Maintaining low intracellular sodium concentrations is vital for almost all organisms. In animals Na^+ efflux is generally governed by Na^+,K^+-ATPase and in fungi by a Na^+-ATPase, called ENA [21, 22]. In the latter, increased activity of Na^+-ATPase with medium alkalinization was observed, suggesting that this enzyme is essential for their survival under high pH environment [21, 22]. Although the ouabain-insensitive Na^+-ATPase has not been cloned so far in mammals, our results suggest that its function may be similar in mammalian cells. Although more studies must be performed to fully clarify our findings, the presence of the Na^+-ATPase may explain the reason why a renal epithelial cell with no Na^+,K^+-ATPase expression is able to maintain its viability.

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