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Overexpression of Na^+/K^+-ATPase Parallels the Increase in Sodium Transport and Potassium Recycling in an *In Vitro* Model of Proximal Tubule Cellular Ageing

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Abstract. Na^+/K^+-ATPase plays a key role in the transport of Na^+ throughout the nephron, but ageing appears to be accompanied by changes in the regulation and localization of the pump. In the present study, we examined the effect of *in vitro* cell ageing on the transport of Na^+ and K^+ ions in opossum kidney (OK) cells in culture. Cells were aged by repeated passing, and Na^+/K^+-ATPase activity and K^+ conductance were evaluated using electrophysiological methods. Na^+K^+-ATPase α_1– and β_1-subunit expression was quantified by Western blot techniques. Na^+/H^+ exchanger activity, changes in membrane potential, cell viability, hydrogen peroxide production and cellular proliferation were determined using fluorimetric assays. *In vitro* cell ageing is accompanied by an increase in transepithelial Na^+ transport, which results from an increase in the number of Na^+/K^+-ATPase α_1- and β_1-subunits, in the membrane. Increases in Na^+/K^+-ATPase activity were accompanied by increases in K^+ conductance as a result of functional coupling between Na^+/K^+-ATPase and basolateral K^+ channels. Cell depolarization induced by both KCl and ouabain was more pronounced in aged cells. No changes in Na^+/H^+ exchanger activity were observed. H_2O_2 production was increased in aged cells, but exposure for 5 days to 1 and 10 μM of H_2O_2 had no effect on Na^+/K^+-ATPase expression. Ouabain (100 nM) increased α_1-subunit, but not β_1-subunit, Na^+/K^+-ATPase expression in aged cells only. These cells constitute an interesting model for the study of renal epithelial cell ageing.

Key words: Renal Na/K-ATPase — Potassium ion channel — Structure/function Na/K-ATPase — Renal Na/H exchange — Membrane transport

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Introduction

The kidney plays an important role in the regulation of blood pressure through modulation of sodium transport across the proximal tubules. Sodium- and potassium-activated adenosine triphosphatase (Na^+/K^+-ATPase), the major Na^+ transporter in renal basolateral epithelia throughout the nephron, is an oligomeric transmembrane protein composed of two main subunits, α and β, the α subunit being the catalytic domain of Na^+/K^+-ATPase (Feraille & Doucet, 2001). This enzyme catalyzes ATP-dependent transport of three sodium ions out of and two potassium ions into the cell per pump cycle, generating a sodium gradient across the cell. It is well established that changes in luminal Na^+ entry proceed in concert with changes in Na^+/K^+-ATPase and K^+ conductance (Beck, Laprade & Lapointe, 1994; Mauerer, Boulpaep & Segal, 1998; Muto et al., 2003). ATP-regulated K^+ channels (K_{ATP}) allow recycling of K^+ taken by Na^+/K^+-ATPase (Warth, 2003). Apical sodium entry stimulates Na^+/K^+-ATPase activity and opening of K_{ATP} channels, which mediate hyperpolarization of the basolateral membrane, facilitating the Na^+ reabsorption and K^+ recycling required for continued Na^+/K^+-ATPase pump turnover (Gomes & Soares-da-Silva, 2003; Noulin et al., 2001; Warth, 2003).

In the kidney, ageing processes cause several structural and functional changes that include a decline in renal blood flow and glomerular filtration rate (Knapowski, Wieczorowska-Tobis & Witowski, 2002; Lindeman, 1990). Several studies have documented deficiencies in the renal dopamine-dependent natriuretic system of old rats (Armando et al., 1995; Fukuda, Bertorello & Aperia, 1991; Kansra, Hussain & Lokhandwala, 1997; Mancini et al., 1991; Meister, Bean & Aperia, 1993; Meister et al., 1992; Soares-da-Silva & Fernandes, 1991; Tenore et al., 1997). In old

Fisher 344 rats, dopamine failed to inhibit renal Na^+/K^+-ATPase activity, which was accompanied by a reduced number of dopamine receptors and defective coupling with G proteins (Asghar, Hussain & Lokhandwala, 2002; Kansra et al., 1997). Overexpression of protein kinase C (PKC)-βI and PKC-δ paralleled an increase in PKC activity in the proximal tubules of old Fisher rats, which may be responsible for hyperphosporylation of Na^+/K^+-ATPase and the diminished dopamine-induced inhibition of Na^+/K^+-ATPase activity (Asghar, Hussain & Lokhandwala, 2003). On the other hand, delocalization of α_1-subunit Na^+/K^+-ATPase in the proximal tubules of aged Fisher rats (Jung et al., 2004) and increases in α_1-subunit Na^+/K^+-ATPase abundance in aged Milan hypertensive rats (Ferrandi et al., 1996) have been reported.

Several groups have reported that ageing of cell lines by serial passing is accompanied by changes in morphology and physiology of the cells (Arthur, 2000; Cottam & Milner, 1997; Huang et al., 2001; Yu, Cook & Sinko, 1997). *In vitro* cellular ageing is considered a useful model for investigating some of the functional changes associated with cellular ageing (Blalock, Porter & Landfield, 1999; Huang et al., 2001; Moon, Cha & Kim, 2003; Porter et al., 1997; Xiong et al., 2004). Established cell lines of renal origin are frequently used as models for analyzing renal transport functions and their regulation. The opossum kidney (OK) renal epithelial cell line was derived from the kidney of a female American opossum and has been widely used as a model of proximal renal tubules (Koyama et al., 1978). OK cells express renal transport systems that are characteristic of proximal tubules and comprise the only renal epithelial cell line possessing high-affinity parathyroid hormone (PTH) receptors coupled to both activation of adenylyl cyclase and inhibition of the Na^+-dependent phosphate cotransporter (Caverzasio, Rizzoli & Bonjour, 1986; Cole et al., 1987; Malmstrom & Murer, 1986; Teitelbaum et al., 1986). Cole and coworkers (1989) described three clonal sublines of OK cells, on the basis of limiting dilution processes, that are morphologically and functionally distinct from parental cells. More recently, our laboratory isolated two different subpopulations of OK cells with origin in the same batch (Gomes et al., 2002).

The aim of the present work was to examine whether *in vitro* cellular ageing induces changes in the ionic transport properties of proximal tubular cells. The major Na^+ transporters on the apical side (Na^+/H^+ exchanger) and on the basolateral side (Na^+/K^+-ATPase) as well as K^+ transport activity were evaluated. Na^+/K^+-ATPase expression and regulation by ouabain and hydrogen peroxide were also evaluated.

Materials and Methods

Cell Culture

OK cells (ATCC 1840 CRL, batch number 2129181), obtained from the American Type Culture Collection (Rockville, MD) at passage 36 of culture, were maintained in a humidified atmosphere of 5% CO_2-95% air at 37°C. Cells were grown in minimum essential medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma), 100 U/ml penicillin G, 0.25 μg/ml amphotericin B, 100 μg/ml streptomycin (Sigma) and 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanosulfonic acid (HEPES, Sigma). The medium was changed every 2 days, and cells reached confluence 3–5 days after initial seeding. For subculturing, the cells were dissociated with 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA, Sigma), split 1:5 and subcultured in a 21-cm^2 growth area (Costar, Badhoevedorp, The Netherlands). Twenty-four hours prior to experiments, the cell medium was free of fetal bovine serum. For electrophysiological studies, the cells were seeded onto polycarbonate filter supports (Snapwell, Costar) at a density of 13,000/well and experiments were performed 4 days after initial seeding.

Cell Proliferation

Cell proliferation was evaluated with the CyQUANT Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR), which produces large fluorescence enhancements upon binding to cellular nucleic acids. OK cells were seeded onto 96-well plates (Costar) at a density of 2,000/well and incubated at 37°C for 9, 24 and 48 h. Plate cultures were harvested by inverting the plates onto paper towels to remove growth medium without disrupting adherent cells and frozen at −70°C. On the day of the experiment, cells were thawed at room temperature and 200 μl of CyQUANT GR dye/lysis buffer (prepared following the manufacturer's protocol) was added to each sample well. Samples were incubated for 5 min at room temperature and protected from light. Fluorescence was measured using a microplate spectrofluorometer (Spectramax Gemini; Molecular Devices, Palo Alto, CA) at excitation and emission wavelengths of 480 and 520 nm, respectively.

Na^+/K^+-ATPase Activity

Na^+/K^+-ATPase activity was examined in OK cells grown on polycarbonate filters (Snapwell, Costar) mounted in Ussing chambers bathed with the Krebs-Henseleit solution, gassed with 5% CO_2-95% O_2 and maintained at 37°C. The standard composition of the Krebs-Hensleit solution was (in mM): NaCl 118, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $CaCl_2$ 2.5 and $MgSO_4$ 1.2, with pH adjusted to 7.4 after gassing with 5% CO_2 and 95% O_2. The apical bathing solution contained mannitol (10 mM) instead of glucose (10 mM) to avoid entry of apical Na^+ through the Na^+-dependent glucose transporter. In Na^+-free Krebs-Henseleit solution, NaCl was replaced with choline chloride and $NaHCO_3$ with choline bicarbonate. In K^+-free Krebs-Henseleit solution, KCl was substituted with cesium chloride. The concentration-response relationship of the short-circuit current (I_{sc}) was determined by bathing the apical cell monolayer with Krebs with increased Na^+ concentrations over the range 0–143 mM without affecting the concentration of other ions. After 5-min stabilization, monolayers were continuously voltage-clamped to zero potential differences by application of an external current, with compensation for fluid resistance, by means of an automatic voltage current clamp (DVC 1000; World Precision Instruments, Sarasota, FL). Cells were

allowed to stabilize for a further 25 min before permeabilization of apical membrane with amphotericin B (1.0 μg/ml); this period was also used for exposure of cells to test compounds. Under these conditions, the resulting I_{sc} is due to Na^+ transport across the basolateral membrane (Gomes, Vieira-Coelho & Soares-da-Silva, 2001). The voltage-current clamp unit was connected to a personal computer by means of a BIOPAC MP1000 data acquisition system (BIOPAC Systems, Goleta, CA). Data were analyzed with Acq-Knowledge 2.0 software (BIOPAC Systems).

Basolateral Membrane K^+ Conductance

Cell monolayers were mounted in Ussing chambers in the presence of an apical to basolateral K^+ gradient (80:5 mM), while the Na^+ concentration was maintained at 25 mM at both sides of the monolayers, gassed with 5% CO_2-95% O_2 and maintained at 37°C. NaCl in the apical solutions was replaced with KCl (75 mM), and NaCl in the basolateral bathing solution was replaced with choline chloride (75 mM). The modified Krebs-Heinseleit solution contained (in mM) 25 NaCl, 5 KCl, 25 choline HCO_3, 1.2 KH_2PO_4, 2.5 $CaCl_2$ and 1.2 $MgSO_4$, with pH adjusted to 7.4. After 5-min stabilization, monolayers were continuously voltage-clamped to zero potential as mentioned above. Ouabain (100 μM) was added to the basolateral bathing solution to inhibit Na^+/K^+-ATPase, and after 25 min apical plasma membrane was permeabilized with amphotericin B (3 μg/ml). Test drugs were usually added to the bath solution 20–30 min before the addition of amphotericin B. The resulting I_{sc} is due to the movement of K^+ (I_K) through channels in the basolateral membrane (DuVall, Guo & Matalon, 1998; Gomes & Soares-da-Silva, 2002a). The concentration-response relationship of the I_K was determined by bathing the basal cell monolayer with Krebs with increased K^+ concentrations over the range 5–80 mM without affecting the concentration of other ions.

Membrane Potential

Changes in membrane potential were monitored with the bisoxonol fluorescent dye bis-(1,3-dibutylbarbituric acid) ($DiBAC_4[3]$). Cells cultured in 96-well plates (Costar) were rinsed twice with assay buffer (in mM: 20 HEPES, 120 NaCl, 2 KCl, 2 $CaCl_2$, 1 $MgCl_2$ and 5 glucose, pH 7.4, at 25°C) containing 5 μM $DiBAC_4(3)$ and then incubated for 30 min in 200 μl buffer solution containing 5 μM $DiBAC_4(3)$ to ensure dye distribution across the cell membrane. Ouabain (100 μM) and KCl (50 mM) were added to the incubation medium, and changes in fluorescence were monitored for 35 min by sampling every 5 s at excitation and emission wavelengths of 488 and 520 nm, respectively. Changes in fluorescence were corrected for background.

Intracellular pH Measurement and Na^+/H^+ Exchanger Activity

Intracellular pH was measured as previously described (Gomes & Soares-da-Silva, 2004; Gomes et al., 2001). At days 4–5 after seeding, cells cultured on 96-well plates (Costar) were incubated at 37°C for 40 min with 5 μM of the acetoxymethyl ester of 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM). Cells were then washed twice with prewarmed dye-free modified Krebs buffer before initiation of the fluorescence recordings. Cells were placed in the sample compartment of a dual-scanning microplate spectrofluorometer (Spectramax Gemini, Molecular Devices), and fluorescence was measured every 19 s, alternating between 440 and 490 nm excitation at 535 nm emission, with a cut-off filter of 530 nm. The ratio of intracellular BCECF fluorescence at 490 and 440 nm was converted to intracellular pH values by comparison with values from an intracellular calibration curve using the nigericin (10 μM) and high-K^+ method (Gomes & Soares-da-Silva, 2004; Gomes et al., 2001).

Na^+/H^+ exchanger activity was assayed as the initial rate of pH_i recovery after an acid load imposed by 20 mM NH_4Cl followed by removal of Na^+ from the Krebs modified buffer solution (in mM: NaCl 140, KCl 5.4, $CaCl_2$ 2.8, $MgSO_4$ 1.2, NaH_2PO_4 0.3, KH_2PO_4 0.3, HEPES 10 and glucose 5, pH 7.4, adjusted with Tris base), in the absence of CO_2/HCO_3 (Gomes & Soares-da-Silva, 2004; Gomes et al., 2001). In these experiments, NaCl was replaced by an equimolar concentration of tetramethylammonium chloride (TMA).

Cell Viability

Cell viability was measured using calcein-AM (Molecular Probes), as previously described (Pedrosa & Soares-da-Silva, 2002). Membrane-permeant calcein-AM, a nonfluorescent dye, is taken up and converted by intracellular esterases to membrane-impermeant calcein, which emits green fluorescence. After treatment, cells were washed twice with Hanks medium (in mM: NaCl 137, KCl 5, $MgSO_4$ 0.8, Na_2HPO_4 0.33, KH_2PO_4 0.44, $CaCl_2$ 0.25, $MgCl_2$ 1.0, Tris HCl 0.15 and sodium butyrate 1.0, pH 7.4) and loaded with 2 μM calcein-AM in Hanks medium at room temperature for 30 min. Fluorescence was measured at 485 nm excitation and 530 nm emission wavelengths in a multiplate reader (Spectramax Gemini). Six wells were treated with ethanol 30 min before calcein-AM addition, and the readings obtained were used for background correction.

Hydrogen Peroxide Production

H_2O_2 was measured fluorometrically using the Amplex™ Red Hydrogen Peroxide Assay Kit (Molecular Probes), as previously described (Pedrosa & Soares-da-Silva, 2002). Amplex Red is a fluorogenic substrate with very low background fluorescence that reacts with H_2O_2 with a 1:1 stoichiometry to produce a highly fluorescent reagent (Mohanty et al., 1997). Measurement of H_2O_2 accumulation was followed in extracellular medium. Fluorescence intensity was measured in a multiplate reader (Spectramax Gemini) at an excitation wavelength of 530 nm and emission wavelength of 590 nm at room temperature. The concentration of H_2O_2 was calculated using a resorufin-H_2O_2 standard calibration curve.

Immunoblotting

OK cells grown for 5 days were rinsed twice with cold phosphate-buffered saline (PBS) and lysed by the addition of radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) containing protease inhibitors: 100 μg/ml phenylmethyl sulfonyl fluoride, 2 μg/ml leupeptin and 2 μg/ml aprotinin. Cells were scraped, briefly sonicated, incubated on ice for 1 h and centrifuged (13,000 rpm for 45 min). Clarified supernatants were used fresh or stored at –70°C. Proteins were mixed with 6x sample buffer (0.35 M Tris-HCl, 4% SDS, 30% glycerol, 9.3% dithiothreitol [pH 6.8], 0.01% bromophenol blue) and warmed at 37°C for 15 min. Equal amounts of total protein (10 μg) were separated on a 7.5% SDS-polyacrylamide gel and electrotransferred to a nitrocellulose membrane in Tris-glycine transfer buffer containing 20% methanol. For the assay of α_1-subunit Na^+/K^+-ATPase abundance, membranes were blocked in 3% nonfat dry milk in PBS-Tween (PBS [pH 7.4] containing 0.05% Tween 20) for 20 min and then incubated overnight at 4°C with anti-α_1-subunit Na^+/K^+-ATPase primary antibody, diluted 1:10,000 in 3%

nonfat dry milk in PBS-Tween (Upstate Biotechnology, Lake Placid, NY). For the assay of β-actin abundance, membranes were blocked in 5% nonfat dry milk in PBS-Tween overnight and then incubated for 1 h with anti β-actin primary antibody, diluted 1:15,000 in 5% nonfat dry milk in PBS-Tween. The immunoblots were subsequently washed, incubated with peroxidase-conjugated secondary antibody and diluted 1:3,000 in 3% nonfat dry milk in PBS-Tween (Santa Cruz Biotechnology, Santa Cruz, CA); the reaction was visualized by enhanced chemiluminescence using the ECL system (Amersham, Arlington Heights, IL). For experiments performed with nontoxic concentrations of H_2O_2 (1 and 10 μM) and ouabain (100 nM), cells were grown for 5 days in the presence of the drug and the medium was changed daily. The densities of the appropriate bands were determined with Quantity one (Bio-Rad, Hercules, CA). For the assay of Na^+/K^+-ATPase β_1-subunit abundance, the membrane was blocked in 3% nonfat dry milk in PBS for 1 h and then incubated overnight at 4°C with a mouse monoclonal anti-β_1-Na^+/K^+-ATPase primary antibody (Santa Cruz Biotechnology), diluted in 3% nonfat dry milk in PBS. The immunoblots were subsequently washed and incubated with 0.5 μg/ml of fluorescently labeled goat anti-mouse secondary antibody (AlexaFluor 680, Molecular Probes) for 90 min at room temperature and protected from light. The membrane was washed and imaged by scanning at 700 nm with an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, Nebraska, USA).

Drugs

Amphotericin B, ouabain, $BaCl_2$ and glybenclamide were purchased from Sigma. BCECF-AM, $DiBAC_4(3)$ and the CyQUANT Cell Proliferation Assay Kit were purchased from Molecular Probes.

Data Analysis

Arithmetic means are given with standard error of the mean (SEM) and geometrical means with 95% confidence values. Statistical analysis was carried out with a one-way analysis of variance followed by Newman-Keuls test for multiple comparisons. $P < 0.05$ was assumed to denote a significant difference.

Results

Cell Proliferation

Over time in culture the same cell line can exhibit different functional characteristics due to the selection of fast-growing clones. In the present study, all experiments were performed with a single batch of relatively young (36 passages) OK cells. Cells used in subsequent evaluations were kept in culture approximately over 30 cell passages, namely from cell passage 40 to 70, which corresponds to 5 months of culture. CyQUANT cell proliferation assay, a fluorescent indicator of cell number based on the fluorescence exhibited by the CyQUANT GR dye upon binding to nucleic acids, was used to determine the proliferative capacity of young and aged OK cells, at passages 40 and 70, respectively. Cells were seeded at equivalent densities on a microplate, and fluorescence was measured 9, 24 and 48 h after seeding. Linear regression analysis of cultured OK cells showed no differences in their ability to proliferate (Fig. 1); the rate of proliferation (arbitrary fluorescence per hour) was 10.0 ± 0.3 and 10.2 ± 1.0 at cell passages 40 and 70, respectively.

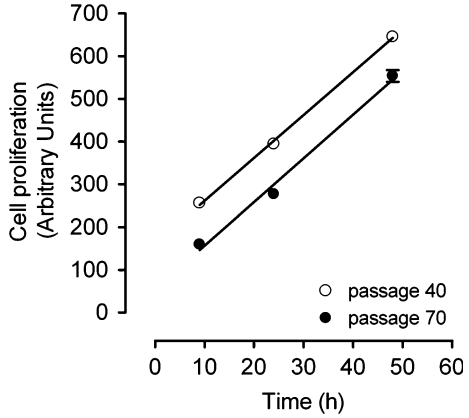

Fig. 1. Cellular proliferation of OK cells in culture using the CyQUANT assay. Young and aged cells were plated at equal density and cultured in standard growth medium for 9, 24 and 48 h. Symbols represent the mean of 24 experiments per group; *lines* show error bars.

Na^+/K^+-ATPase Activity and Expression

Na^+/K^+-ATPase activity was evaluated using an electrophysiological method in which cell monolayers were continuously monitored for changes in I_{sc} after the addition of amphotericin B (1.0 μg/ml) to the apical cell side to increase the Na^+ delivered to Na^+/K^+-ATPase. The amphotericin B (1.0 μg/ml)-induced increase in I_{sc} was inhibited by ouabain (100 μM) and by removal of apical Na^+ (NaCl replaced with choline chloride and $NaHCO_3$ with choline bicarbonate) by 79% and 92%, respectively (Fig. 2). Removal of K^+ from the solution bathing the basolateral cell side (substitution by cesium chloride) markedly attenuated (87% reduction) the amphotericin B-induced increase in I_{sc}. However, blockade of basolateral K^+ channels with $BaCl_2$ (1 mM) applied from the basolateral cell side failed to affect the amphotericin B-induced increase in I_{sc} (Fig. 2). As shown in Figure 3, the increase in I_{sc} (in $\mu A/cm^2$) induced by 0.6 and 1.0 μg/ml amphotericin B increased with the number of cell passages and was, therefore, largely dependent on the age of the cell line. However, 0.3 μg/ml amphotericin B did not increase I_{sc} at all cell passages (Fig. 3). The relationship between Na^+/K^+-ATPase activity and intracellular Na^+ was also studied. The amphotericin B-induced increase in I_{sc} was measured at increasing concentrations of Na^+ (0–143 mM) (NaCl was replaced by equimolar concentration of choline choloride). At passages 50 and 60, the amphotericin B-induced increase in I_{sc} was Na^+-dependent and saturable while

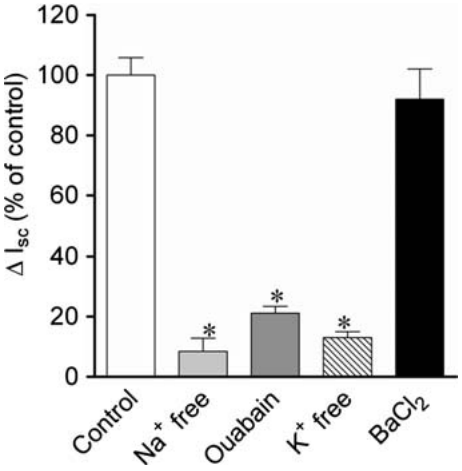

Fig. 2. Effect of Na^+ removal from the solution bathing the apical side and K^+ from the solution bathing the basolateral side and effects of ouabain and $BaCl_2$ upon changes in short-circuit current (ΔI_{sc}, % of control) induced by amphotericin B (1.0 μg/ml) applied from the apical cell side. *Columns* represent the mean of five experiments per group; *vertical lines* show error bars. *Significant differences from the control ($P < 0.05$).

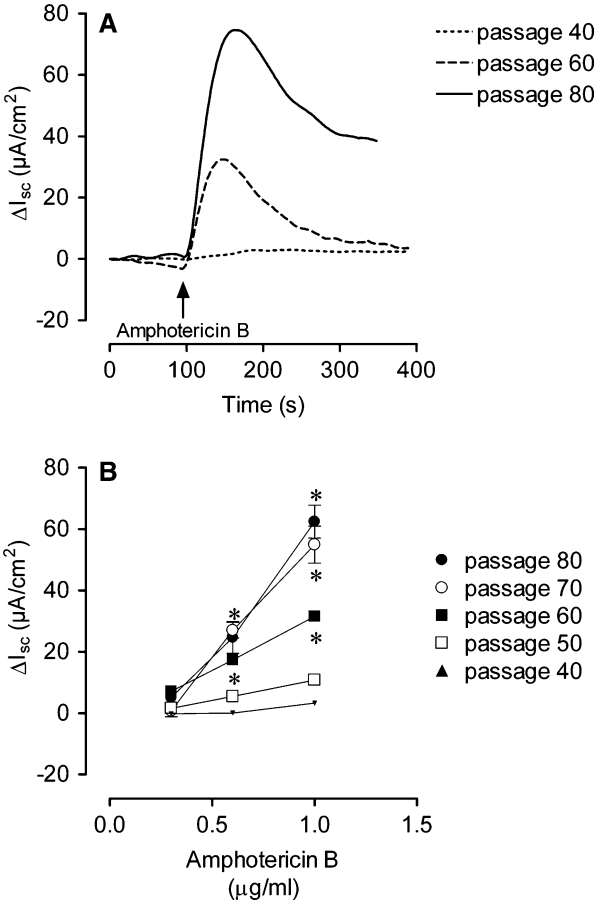

Fig. 3. (*A*) I_{Na} traces for the effect of amphotericin B (1.0 μg/ml) at passages 40, 60 and 80. (*B*) Mean values of amphotericin B (0.3, 0.6 and 1.0 μg/ml)-induced increases in I_{sc} across OK cell monolayers at passages 40, 50, 60, 70 and 80. Symbols represent the mean of 5–12 experiments per group; *vertical lines* show error bars. *Significant differences from passage 40 ($P < 0.05$).

approaching 100 mM Na^+ (Fig. 4). As depicted in Figure 4 and Table 1, cell ageing was accompanied by an increase in V_{max} values without affecting the affinity of Na^+/K^+-ATPase for Na^+. These results suggest that the differences observed in basolateral Na^+ transport may not be due to an altered affinity of Na^+/K^+-ATPase for Na^+ but probably results from an increase in the number of pump units in the membrane. To evaluate this possibility, the expression of the α_1- and β_1-subunits of Na^+/K^+-ATPase was quantified. In agreement with the functional data, the results obtained from four independent experiments show that the abundance of α_1- and β_1-subunits of Na^+/K^+-ATPase increased with the number of cell passages (Fig. 5A and B); this was particularly evident in cells with high number of cell passages (60–80 passages). The relative abundance of α_1- and β_1-subunits of Na^+/K^+-ATPase were four and three times greater after 40 cell passages, respectively. However, the ratio between α_1- and β_1-subunits of Na^+/K^+-ATPase did not attain statistical significance (Fig. 5C). The antibodies raised against the α_1- and β_1-subunits of Na^+/K^+-ATPase revealed the presence of, respectively, ~100-kDa and ~50-kDa bands.

K^+ Conductance

K^+ channels located at the basolateral membrane of proximal tubules are functionally coupled to Na^+/K^+-ATPase (Beck et al., 1994; Gomes & Soares-da-Silva, 2002a; Warth, 2003). Thus, the next set of experiments was aimed at evaluating K^+ conductance in aged OK cells. In the presence of an apical to basolateral K^+ gradient (80:5 mM), addition of amphotericin B (3 μg/ml) to the apical bathing solution induced an increase in I_K. The increase in I_K was largely due to K^+ transport across the basolateral membrane, as revealed by marked inhibition of the amphotericin B-induced increase in K^+ conductance by the nonspecific K^+ channel blocker $BaCl_2$ (1 mM, 78% reduction) and by pretreatment with the ATP-sensitive channel blocker glibenclamide (10 μM, 30% reduction) (Fig. 6). The amphotericin B-induced increase in K^+ conductance was dependent on the age of the cells and increased with the number of cell passages (Fig. 7).

Membrane Potential

Since the primary role of K_{ATP} channels is to recycle K^+ pumped into the cell by Na^+/K^+-ATPase, thus maintaining the plasma membrane potential, changes in membrane potential were determined after treatment with KCl (50 mM) and ouabain (100 μM). The fluorescent dye $DiBAC_4(3)$ was used to monitor

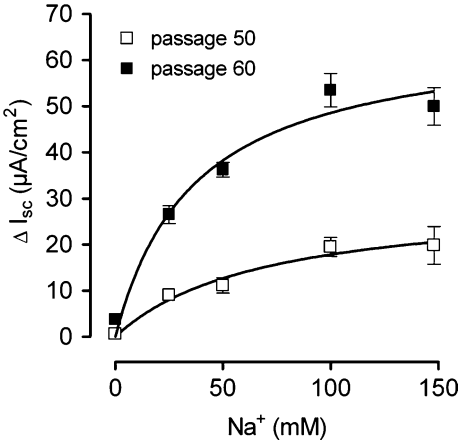

Fig. 4. Na^+ dependence of amphotericin B (1.0 μg/ml)-induced increases in short-circuit current (ΔI_{sc}, $\mu A/cm^2$) at passages 50 and 60. Symbols represent the mean of five experiments per group; *vertical lines* show error bars.

Table 1. Apparent K_m and V_{max} for Na^+K^+-ATPase activity at passages 50 and 60

	K_m (mM)	V_{max}($\mu A/cm^2$)
Passage 50	68.5 ± 26.8	30.1 ± 5.2
Passage 60	36.9 ± 15.1	66.4 ± 8.8*

Values are means ± SEM of five experiments per group. *Significant differences from passage 50 ($P < 0.05$).

changes in membrane potential. As shown in Figure 8A and B, both KCl and ouabain produced cell depolarization, as evidenced by time-dependent increases in cell fluorescence. Cell depolarization was also dependent on the number of cell passages (Fig. 8C).

Na^+/H^+ Exchanger Activity

Because Na^+ pump activity was augmented in aged OK cells, we hypothesized that the resulting increase in the gradient for Na^+ transepithelial flux might alter the activity of the apical Na^+/H^+ exchanger, the main mechanism responsible for the apical transport of Na^+ in renal proximal tubular cells. Na^+/H^+ exchanger activity was assayed as the initial rate of pH_i recovery measured after an acid load imposed by 20 mM NH_4Cl followed by removal of Na^+ from the Krebs modified buffer solution, in the absence of CO_2/HCO_3. As shown in Table 2, the pH_i recovery rates (in ΔpH units/s) during the linear phase of pH_i recovery after intracellular acidification did not change with the number of cell passages.

H_2O_2 production and Na^+,K^+-ATPase Expression

Free radicals and other reactive products of oxygen metabolism were suggested to contribute to age-related cellular changes (Knapowski et al., 2002; Lavrovsky et al., 2000). It was, therefore, felt worthwhile to evaluate H_2O_2 production and examine the influence on the expression of α_1-subunit Na^+/K^+-ATPase. H_2O_2 levels in the extracellular medium were significantly higher in aged OK cells, being 231.4 ± 3.5 and 309.4 ± 2.3 nM at passages 51 and 73, respectively (Table 3). In order to determine if H_2O_2 might influence Na^+/K^+-ATPase expression, OK cells were treated for 5 days with 1 and 10 μM H_2O_2. As shown in Figure 9A, cell viability was not compromised after incubation with H_2O_2 up to 10 μM. The relative abundance of α_1-subunit Na^+/K^+-ATPase was not affected by prolonged treatment with H_2O_2 (Fig. 9B).

Ouabain and Na^+/K^+-ATPase Expression

Treatment of renal cells with nanomolar concentrations of ouabain has been shown to stimulate Na^+/K^+-ATPase activity (Ferrari et al., 1998; 1999; 2003). In the next series of experiments, both young (passage 40–50) and aged (passage 60–80) OK cells were treated for 5 days with a nontoxic (Fig. 10A) concentration of ouabain (100 nM) and the abundance of α_1- and β_1-subunits of Na^+/K^+-ATPase was determined. As revealed by immunoblotting, only in aged cells was the abundance of α_1-subunit Na^+/K^+-ATPase increased following treatment with ouabain (Fig. 10B). However, the abundance of β_1-subunit Na^+/K^+-ATPase was not altered following treatment with ouabain (Fig. 10C).

Discussion

Changes in functional characteristics of cell lines can be due to the accumulation of mutations, changes in the pattern of protein expression, posttranslational modifications or selection of different populations of cells (Arthur, 2000; Cottam & Milner, 1997; Huang et al., 2001; Yu et al., 1997). Previous studies have reported the isolation of subpopulations of OK cells based on limiting dilutions; these cell populations were functionally distinct from the parental cell line and from each other (Cole et al., 1989; Gomes et al., 2002). The increase in Na^+/K^+-ATPase activity and K^+ conductance reported here was a consistent observation in different series of cells with high number of cell passages, suggesting that the results are due to alterations induced by repetitive cell passing. Changes observed in aged OK cells may not be due to selection of fast growing populations since young and aged OK cells have identical abilities to proliferate. The finding that 0.3 μg/ml amphotericin B failed to elicit Na^+ currents at all cell passages suggests that the presence of amphotericin B (0.25 μg/ml) in the culture medium had no impact on

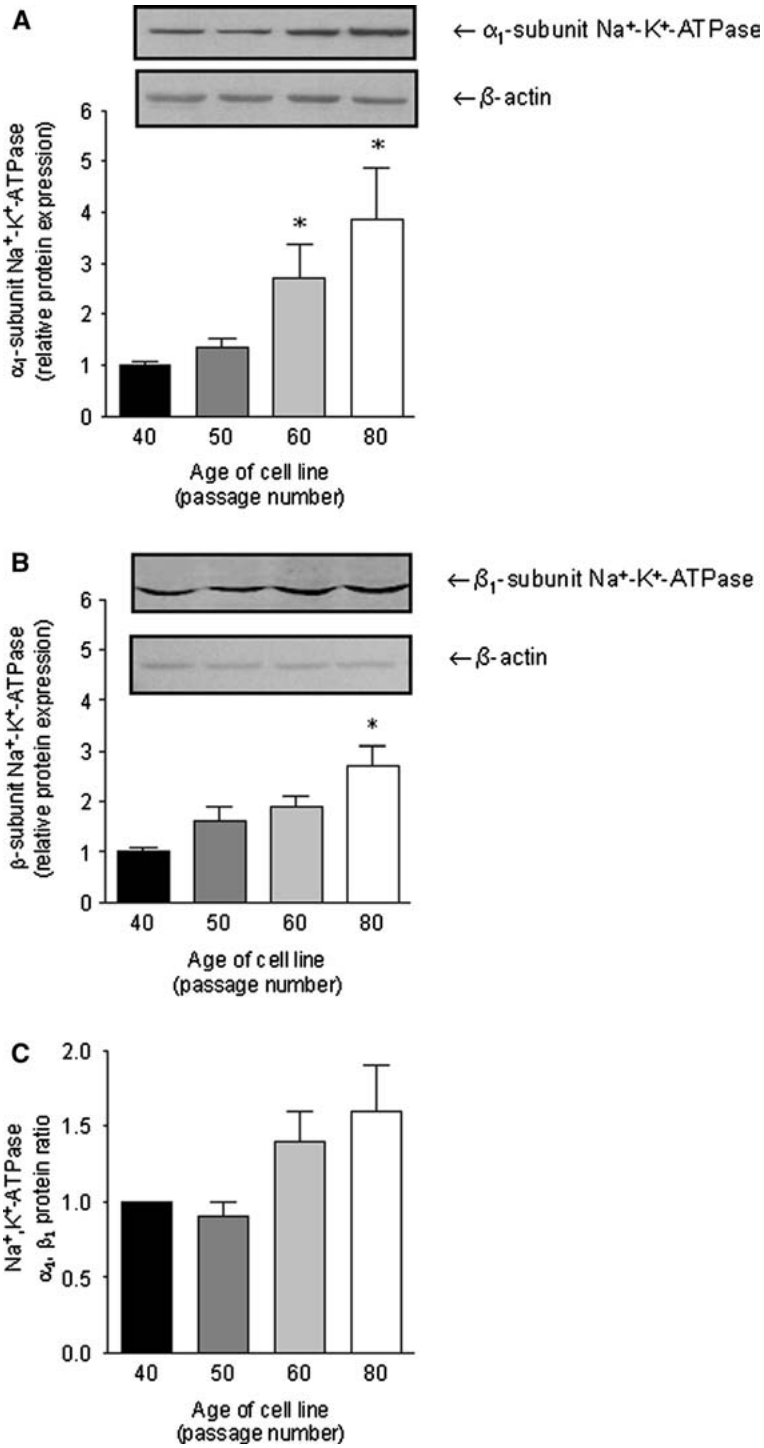

Fig. 5. Abundance of (*A*) α_1-subunit and (*B*) β_1-subunit Na^+/K^+-ATPase expression in OK cell monolayers. Each blot was equally loaded with 10 μg of total protein. Immunoblots were repeated four times and normalized for the expression of β-actin. (*C*) Ratio between protein expression of α_1- and β_1-subunits of Na^+/K^+-ATPase. *Columns* indicate relative density and represent the mean of four separate experiments; *vertical lines* show error bars. *Significant differences from passages 40 and 50 ($P < 0.05$).

the increase in Na^+/K^+-ATPase activity and α_1- and β_1-subunits of Na^+/K^+-ATPase expression with cell passaging.

The results obtained in the present study show that Na^+/K^+-ATPase activity, but not Na^+/H^+ exchanger activity, increased with the number of cell passages. The increase in Na^+/K^+-ATPase activity paralleled the increment in Na^+/K^+-ATPase present in the cellular membrane, rather than changes in Na^+/K^+-ATPase affinity for Na^+, as shown by the increase in V_{max} with cell passages without changes in K_m values. These changes can be due to posttranslational modifications that interfere with the number of pump units in the cell surface, alterations in protein expression pattern or both. Immunoblotting studies revealed an increase in Na^+/K^+-ATPase subunit

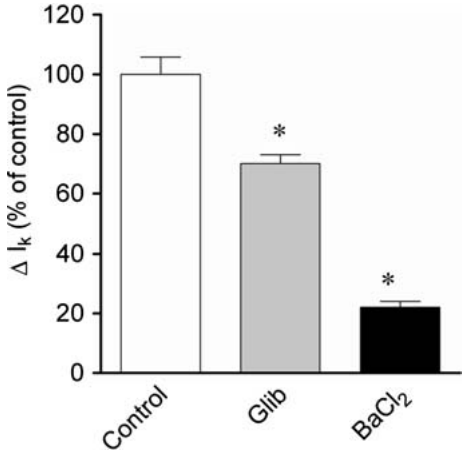

Fig. 6. Effect of glibenclamide (*Glib*, 10 μM) and barium chloride ($BaCl_2$, 1 mM) upon changes in K^+ conductance (ΔI_K, % of control) induced by amphotericin B (3.0 μg/ml) applied from the apical cell side. Columns represent the mean of five experiments per group; *vertical lines* show error bars. *Significant differences from control ($P < 0.05$).

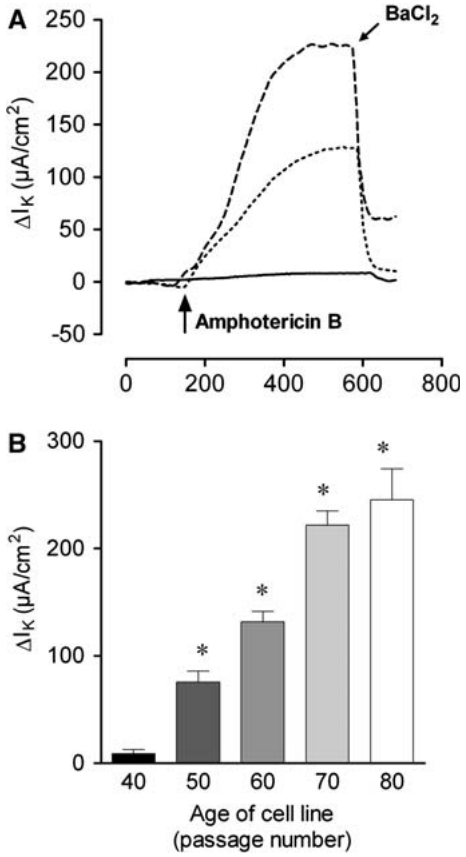

Fig. 7. (*A*) I_K for the effect of amphotericin B (3.0 μg/ml) at passages 40, 60 and 80. Barium chloride ($BaCl_2$, 1 mM) was added after current reached the plateau. (*B*) Amphotericin B (3.0 μg/ml)-induced increases in K^+ conductance (ΔI_K, $\mu A/cm^2$) across aged OK cell monolayers at passages 40, 50, 60, 70 and 80. *Columns* represent the mean of five experiments per group; *vertical lines* show error bars. *Significant differences from passage 40 ($P < 0.05$).

expression that may account for the differences observed in Na^+/K^+-ATPase activity. In this respect, it is interesting to underline the observation that the abundance of β_1-subunit Na^+/K^+-ATPase was less pronounced. One possible explanation for this finding is that the preexisting pool of β_1-subunits was excessive and available to form functional heterodimers with the overexpressed α_1-subunit. The observation in transfected cells of increased basolateral Na^+ currents mediated by Na^+/K^+-ATPase also implies that the α-subunits were assembled with their respective β-subunits in the endoplasmic reticulum, followed the secretory pathway, and then the newly synthesized proteins were correctly sorted to the basolateral membrane.

The regulation of Na^+/K^+-ATPase is of particular importance to maintain sodium homeostasis. The majority of studies have addressed Na^+/K^+-ATPase regulation through phosphorylation of α_1-subunit mediated by protein kinases C and A (Gomes & Soares-da-Silva, 2002b; Pedemonte et al., 1997; Pierre et al., 2002). Phosphorylation results from the activation of a cascade of intracellular mechanisms by hormones such as dopamine, norepinephrine and insulin; growth factors; and more directly by ionic distribution across the membrane (Efendiev et al., 2000; 2005; Feraille & Doucet, 2001). However, there is limited information on the transcriptional regulation of the α_1-subunit of the Na^+/K^+-ATPase gene, namely in ageing. Aberrant staining of α_1-subunit Na^+/K^+-ATPase in basolateral membranes and some apical staining in proximal tubules of aged (24 months) Fisher 344 rats have been observed (Jung et al., 2004). In Milan normotensive and hypertensive rats, ageing has been found to be accompanied by increases in renal Na^+/K^+-ATPase activity and α_1 and β_1 protein expression. In Milan hypertensive rats, the increase in renal Na^+/K^+-ATPase activity and α_1 and β_1 protein expression is already present before the development of hypertension (Ferrandi et al., 1996). Although the observations made in this model of *in vitro* cellular ageing do not completely resemble those observed in old rats, studies on these cells may prove useful for the understanding of Na^+/K^+-ATPase regulation *in vivo*, which in some cases leads to the development of hypertension.

Age-dependent upregulation of Na^+/K^+-ATPase observed both *in vitro* and *in vivo* can be due to many factors, including increased apical sodium entrance, positive feedback due to inhibition of basolateral sodium extrusion by ouabain, hormonal steroid activation and alterations in cytoskeletal proteins. Na^+/K^+-ATPase gene and protein expression stimulation by low K^+ in clonal MDCK cells has been reported to require an increase in reactive oxygen species (ROS) production (Zhou et al., 2003).

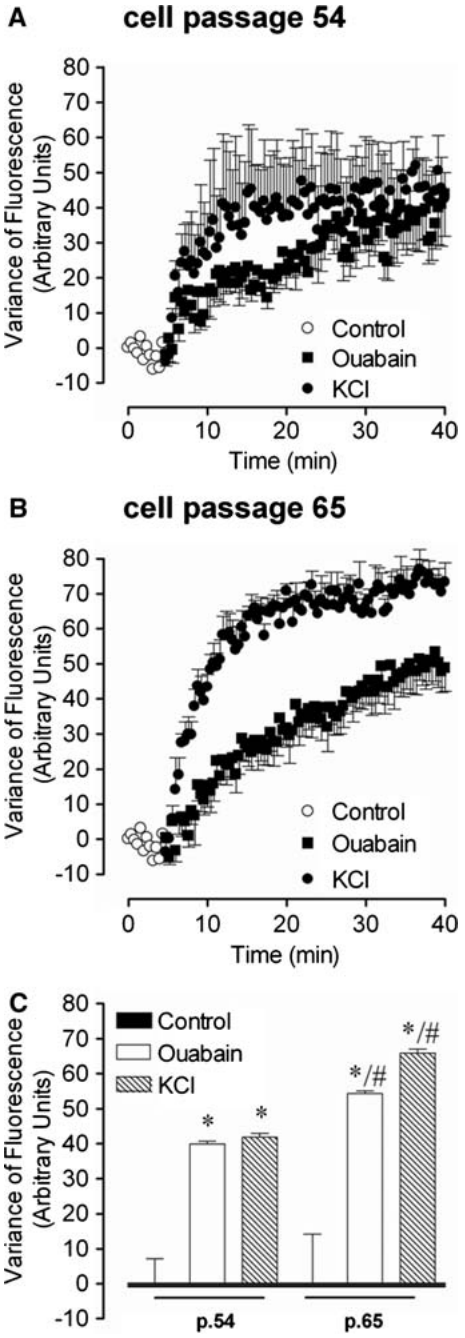

Fig. 8. Representative traces (*A*, passage 54; *B*, passage 65) and summarized data (***C***) of the effect of ouabain (100 μM) and KCl (50 mM) on changes in $DiBAC_4(3)$ fluorescence in OK cells. Compounds were added after a baseline period of 5 min. Fluorescence sampling was done every 5 s. Symbols represent the mean of five experiments per group; *vertical lines* show error bars. *Significant differences from the control ($P < 0.05$). #Significant differences from passage 54 ($P < 0.05$).

The contribution of some of these factors to the changes observed was addressed in our study.

ROS production has been associated with the activation of several signaling pathways and is known to play a significant role in *in vivo* ageing (Finkel, 2003; Knapowski et al., 2002). In fact, we have observed higher concentrations of H_2O_2 production in aged OK cells. However, exposure of OK cells for 5 days to nontoxic concentrations of H_2O_2 failed to alter Na^+/K^+-ATPase protein expression. ROS are generated as by-products of cellular metabolism, namely by mitochondria during ATP formation. Since Na^+/K^+-ATPase utilizes 10–30% of cellular ATP, an increase in Na^+/K^+-ATPase activity is expected to be accompanied by an increase in ATP production. The relationship between Na^+/K^+-ATPase and ATP may justify differences in H_2O_2 levels between new and old OK cells. In this model, increased accumulation of H_2O_2 in extracellular medium seems to be a consequence, rather than a cause, of the increase in Na^+/K^+-ATPase activity in OK aged cells.

Table 2. Apical Na^+/H^+ exchanger activity in OK cells at passages 40, 50, 60 and 70

	Na^+/H^+ exchanger activity (ΔpH units/s)
Passage 40	0.00465 ± 0.00016
Passage 50	0.00447 ± 0.00011
Passage 60	0.00407 ± 0.00014
Passage 70	0.00453 ± 0.00010

Values are means ± SEM of five experiments per group.

Increases in Na^+/K^+-ATPase activity have been observed after incubation of cells with low concentrations of ouabain (Ferrari et al., 1998; 1999; 2003). Ouabain binds to Na^+/K^+-ATPase and activates several pathways that ultimately may regulate gene expression (Ferrari et al., 1998; 1999; 2003). In cardiac myocytes, some of these events include increases in ROS production, increases in $[Ca^{2+}]_i$, activation of several transcription factors related to early response genes and growth-mediated genes, stimulation of protein synthesis and nyocyte hypertrophy (Aydemir-Koksoy, Abramowitz & Allen, 2001; Xie & Askari, 2002). The role Na^+/K^+-ATPase as an intracellular signal-transducing molecule, namely as a consequence of the interaction with ouabain, has been demonstrated in other cell types and is independent of changes in intracellular Na^+ and K^+ concentrations (Aydemir-Koksoy et al., 2001; Xie & Askari, 2002). The results presented here show an increase in Na^+/K^+-ATPase expression following treatment with ouabain (100 nM) over 5 days in aged cells only. An ouabain-like factor (OLF) has been isolated from the plasma and seems to play a role in the control of Na^+ homeostasis and the develoment of hypertension (Manunta et al., 2001; Vakkuri et al., 2001). The prolonged infusion of ouabain has also been shown to induce reversible hypertension (Ferrari et al., 1998; 2003; Manunta et al., 1994; Tian, Dang & Lu, 2001). At this stage, we cannot rule out

Table 3. Levels of H_2O_2 in the extracellular medium in young (passage 40–50) and old (passage 60–80) OK cells

	H_2O_2 (nM)
Young	236.5 ± 2.7
Old	319.3 ± 2.9*

Values are means ± SEM of 24 experiments per group. *Significant differences from young OK cells ($P < 0.05$).

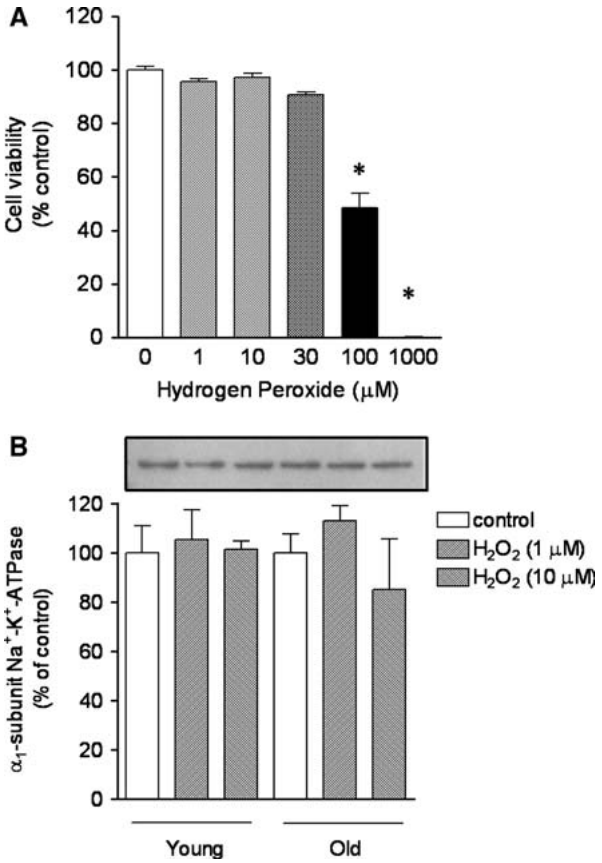

Fig. 9. (*A*) Concentration-dependent effect of H_2O_2 on cell viability. (*B*) Relative abundance of α_1-subunit Na^+/K^+-ATPase expression in young (passage 40–50) and aged (passage 60–80) OK cell monolayers treated with 1 and 10 μM of H_2O_2 for 5 days. Each blot was equally loaded with 10 μg of total protein. *Columns* represent the mean of four experiments; *vertical lines* show error bars. *Significant differences from control ($P < 0.05$).

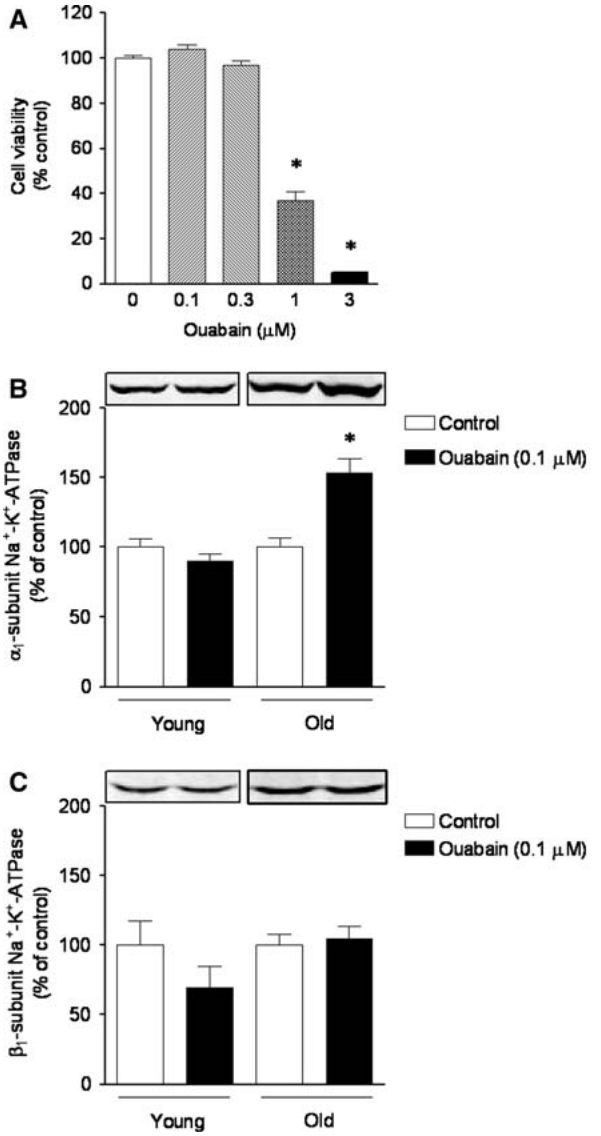

Fig. 10. (*A*) Concentration-dependent effect of ouabain on cell viability. Relative abundance of (*B*) α_1-subunit Na^+/K^+-ATPase and (*C*) β_1-subunit Na^+/K^+-ATPase expression in both young (passage 40–50) and aged (passage 60–80) OK cell monolayers treated with ouabain (100 nM) for 5 days. Each blot was equally loaded with 10 μg of total protein. Immunoblots were repeated four times and normalized for the expression of β-actin. *Columns* represent the mean of four experiments; *vertical lines* show error bars. *Significant differences from the control ($P < 0.05$).

the possibility that fetal bovine serum added to the culture medium contains OLF and that the changes observed in OK cells over time in culture might, in part, be induced by permanent exposure to OLF. This hypothesis is currently being addressed.

Several studies have reported that the functional coupling of Na^+/K^+-ATPase and K^+ conductance is crucial for sustaining transport in the proximal tubules (Beck et al., 1994; Mauerer et al., 1998; Muto et al., 2003; Noulin et al., 2001; Warth, 2003). To maintain a constant intracellular K^+ concentration, alterations in Na^+/K^+-ATPase must be compensated by alterations in K^+ conductance in order to maintain intracellular K^+ at normal values. It is interesting to note that increases in Na^+/K^+-ATPase activity after serial cell passing were accompanied by parallel increases in K^+ conductance. Opening of basolateral K_{ATP} channels mediates membrane hyperpolarization, which facilitates Na^+ reabsorption and K^+ recycling at the basolateral membrane.

Cell depolarization with KCl (50 mM) increased membrane potential, as evidenced by increases in cell fluorescence to $DiBAC_4(3)$. Inhibition of Na^+/K^+-ATPase activity after ouabain treatment also produced cell membrane depolarization, in agreement with the view that changes in Na^+/K^+-ATPase activity alter K^+ conductance and membrane potential. As the aged cells used in the present study were endowed with increased Na^+/K^+-ATPase activity and K^+ conductance, it was expected that the magnitude of changes induced in membrane potential would also be dependent on the age of the cell line. In fact, a ouabain- and KCl-induced increase in membrane potential was more pronounced in cells with higher passages and increased pump-leak coupling activity.

The absence of correlation between increases in Na^+/K^+-ATPase and Na^+/H^+ exchanger activities in the present study contrasts with the findings previously reported by our group in OK cells engineered to overexpress Na^+/K^+-ATPase (Gomes & Soares-da-Silva, 2005). These OK cells were transfected with a cDNA construct encoding the rat Na^+/K^+-ATPase α_1-subunit. Permanent cell lines derived by transfection of rat α_1-subunit cDNA overexpress the Na^+ pump as well as the apical type 3 Na^+/H^+ exchanger, and these changes are accompanied by increases in the activities of both transporters (Gomes & Soares-da-Silva, 2005). On the other hand, we previously reported the isolation of two clonal subpopulations of OK cells (OK_{LC} and OK_{HC}) with origin in the same batch from the American Type Culture Collection (Gomes et al., 2002), in which the most impressive differences between OK_{LC} and OK_{HC} cells were the overexpression of both Na^+/K^+-ATPase and type 3 Na^+/H^+ exchanger by the latter, accompanied by increased activities of these transporters. It is unlikely that the absence of correlation between increases in Na^+/K^+-ATPase and Na^+/H^+ exchanger activities may be related to differences in the magnitude of increases in Na^+/K^+-ATPase activity. In fact, the two- to threefold increase in Na^+/K^+-ATPase activity observed in aged cells (*vs.* young cells) is similar to that observed in OK_{HC} cells (*vs.* OK_{LC} cells) and OK cells transfected with the rat α_1-subunit Na^+/K^+-ATPase (*vs.* non-transfected cells). It is suggested that the process of cellular ageing may interfere with mechanisms that regulate the expression or the function of the Na^+/H^+ exchanger and prevent it from adapting to increases in Na^+/K^+-ATPase activity, as occurred in OK_{LC} and OK_{HC} cells and OK cells overexpressing the rat α_1-subunit Na^+/K^+-ATPase. Studies are ongoing to evaluate this possibility.

In conclusion, *in vitro* cellular ageing of OK cells is accompanied by an increase in basolateral Na^+ transport, which results from an increase in the number of operational pump units, as revealed by the increase in α_1- and β_1-subunits of Na^+/K^+-ATPase expression, without changes in the affinity of the pump for intracellular Na^+. The ouabain-induced increase in α_1-subunit Na^+/K^+-ATPase in aged cells may result from effects of ouabain at the sodium pump gene expression. Increases in Na^+/K^+-ATPase activity in OK cells were accompanied by increases in K^+ conductance, as a result of functional coupling between Na^+/K^+-ATPase and basolateral K^+ channels, without apparent consequences at the level of the apical Na^+/H^+ exchanger.

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References

Armando, I., Nowicki, S., Aguirre, J., Barontini, M. 1995. A decreased tubular uptake of dopa results in defective renal dopamine production in aged rats. *Am. J. Physiol.* **268:**F1087–F1092

Arthur, J.M. 2000. The MDCK cell line is made up of populations of cells with diverse resistive and transport properties. *Tissue Cell* **32:**446–450

Asghar, M., Hussain, T., Lokhandwala, M.F. 2002. Higher basal serine phosphorylation of D1A receptors in proximal tubules of old Fischer 344 rats. *Am. J. Physiol.* **283:**F350–F355

Asghar, M., Hussain, T., Lokhandwala, M.F. 2003. Overexpression of PKC-betaI and -delta contributes to higher PKC activity in the proximal tubules of old Fischer 344 rats. *Am. J. Physiol.* **285:**F1100–F1107

Aydemir-Koksoy, A., Abramowitz, J., Allen, J.C. 2001. Ouabain-induced signaling and vascular smooth muscle cell proliferation. *J. Biol. Chem.* **276:**46605–46611

Beck, J.S., Laprade, R., Lapointe, J.Y. 1994. Coupling between transepithelial Na transport and basolateral K conductance in renal proximal tubule. *Am. J. Physiol.* **266:**F517–F527

Blalock, E.M., Porter, N.M., Landfield, P.W. 1999. Decreased G-protein-mediated regulation and shift in calcium channel types with age in hippocampal cultures. *J. Neurosci.* **19:**8674–8684

Caverzasio, J., Rizzoli, R., Bonjour, J.P. 1986. Sodium-dependent phosphate transport inhibited by parathyroid hormone and cyclic AMP stimulation in an opossum kidney cell line. *J. Biol. Chem.* **261:**3233–3237

Cole, J.A., Eber, S.L., Poelling, R.E., Thorne, P.K., Forte, L.R. 1987. A dual mechanism for regulation of kidney phosphate transport by parathyroid hormone. *Am. J. Physiol.* **253:**E221–E227

Cole, J.A., Forte, L.R., Krause, W.J., Thorne, P.K. 1989. Clonal sublines that are morphologically and functionally distinct from parental OK cells. *Am. J. Physiol.* **256:**F672–F679

Cottam, D.M., Milner, M.J. 1997. Effect of age on the growth and response of a *Drosophila* cell line to moulting hormone. *Tissue Cell* **29:**727–732

DuVall, M.D., Guo, Y., Matalon, S. 1998. Hydrogen peroxide inhibits cAMP-induced Cl^- secretion across colonic epithelial cells. *Am. J. Physiol.* **275:**C1313–C1322

Efendiev, R., Bertorello, A.M., Pressley, T.A., Rousselot, M., Feraille, E., Pedemonte, C.H. 2000. Simultaneous phosphorylation of Ser11 and Ser18 in the alpha-subunit promotes the recruitment of Na^+,K^+-ATPase molecules to the plasma membrane. *Biochemistry* **39:**9884–9892

Efendiev, R., Chen, Z., Krmar, R.T., Uhles, S., Katz, A.I., Pedemonte, C.H., Bertorello, A.M. 2005. The 14-3-3 protein translates the NA^+,K^+-ATPase α1-subunit phosphorylation signal into binding and activation of phosphoinositide 3-kinase during endocytosis. *J. Biol. Chem.* **280:**16272–16277

Feraille, E., Doucet, A. 2001. Sodium-potassium-adenosinetriphosphatase-dependent sodium transport in the kidney: hormonal control. *Physiol. Rev.* **81:**345–418

Ferrandi, M., Tripodi, G., Salardi, S., Florio, M., Modica, R., Barassi, P., Parenti, P., Shainskaya, A., Karlish, S., Bianchi, G., Ferrari, P. 1996. Renal Na,K-ATPase in genetic hypertension. *Hypertension* **28:**1018–1025

Ferrari, P., Ferrandi, M., Torielli, L., Barassi, P., Tripodi, G., Minotti, E., Molinari, I., Melloni, P., Bianchi, G. 2003. Antihypertensive compounds that modulate the Na-K pump. *Ann. N. Y. Acad. Sci.* **986:**694–701

Ferrari, P., Ferrandi, M., Tripodi, G., Torielli, L., Padoani, G., Minotti, E., Melloni, P., Bianchi, G. 1999. PST 2238: A new antihypertensive compound that modulates Na,K-ATPase in genetic hypertension. *J. Pharmacol. Exp. Ther.* **288:**1074–1083

Ferrari, P., Torielli, L., Ferrandi, M., Padoani, G., Duzzi, L., Florio, M., Conti, F., Melloni, P., Vesci, L., Corsico, N., Bianchi, G. 1998. PST2238: A new antihypertensive compound that antagonizes the long-term pressor effect of ouabain. *J. Pharmacol. Exp. Ther.* **285:**83–94

Finkel, T.. 2003. Oxidant signals and oxidative stress. *Curr. Opin. Cell Biol.* **15:**247–254

Fukuda, Y., Bertorella, A., Aperia, A.. 1991. Ontogeny of the regulation of Na^+,K^+-ATPase activity in the renal proximal tubule cell. *Pediatr. Res.* **30:**131–134

Gomes, P., Soares-da-Silva, P. 2002a. D_2-like receptor-mediated inhibition of Na^+/K^+-ATPase activity is dependent on the opening of K^+ channels. *Am. J. Physiol.* **283:**F114–F123

Gomes, P., Soares-da-Silva, P. 2002b. Role of cAMP-PKA-PLC signaling cascade on dopamine-induced PKC-mediated inhibition of renal Na^+/K^+-ATPase activity. *Am. J. Physiol.* **282:**F1084–F1096

Gomes, P., Soares-da-Silva, P. 2003. Dopamine D_2-like receptor-mediated opening of K^+ channels in opossum kidney cells. *Br. J. Pharmacol.* **138:**968–976

Gomes, P., Soares-da-Silva, P. 2004. Dopamine acutely decreases type 3 Na^+/H^+ exchanger activity in renal OK cells through the activation of protein kinases A and C signalling cascades. *Eur. J. Pharmacol.* **488:**51–59

Gomes, P., Soares-da-Silva, P. 2006. Up-regulation of apical NHE3 in renal OK cells overexpressing the rodent alpha1-subunit of the Na+ pump. *Am. J. Physiol.* **290**:R1142–R1150

Gomes, P., Vieira-Coelho, M.A., Soares-da-Silva, P. 2001. Ouabain-insensitive acidification by dopamine in renal OK cells: Primary control of the Na^+/H^+ exchanger. *Am. J. Physiol.* **281:**R10–R18

Gomes, P., Xu, J., Serrao, P., Doria, S., Jose, P.A., Soares-da-Silva, P. 2002. Expression and function of sodium transporters in two opossum kidney cell clonal sublines. *Am. J. Physiol.* **283:**F73–F85

Huang, W., Carlsen, B., Rudkin, G.H., Shah, N., Chung, C., Ishida, K., Yamaguchi, D.T., Miller, T.A. 2001. Effect of serial passage on gene expression in MC3T3-E1 preosteoblastic cells: A microarray study. *Biochem. Biophys. Res. Commun.* **281:**1120–1126

Jung, K.Y., Dean, D., Jiang, J., Gaylor, S., Griffith, W.H., Burghardt, R.C., Parrish, A.R. 2004. Loss of N-cadherin and alpha-catenin in the proximal tubules of aging male Fischer 344 rats. *Mech. Ageing Dev.* **125:**445–453

Kansra, V., Hussain, T., Lokhandwala, M.F. 1997. Alterations in dopamine DA1 receptor and G proteins in renal proximal tubules of old rats. *Am. J. Physiol.* **273:**F53–F59

Knapowski, J., Wieczorowska-Tobis, K., Witowski, J. 2002. Pathophysiology of ageing. *J. Physiol. Pharmacol.* **53:**135–146

Koyama, H., Goodpasture, C., Miller, M.M., Teplitz, R.L., Riggs, A.D. 1978. Establishment and characterization of a cell line from the American opossum (*Didelphys virginiana*). *In Vitro* **14:**239–246

Lavrovsky, Y., Chatterjee, B., Clark, R.A., Roy, A.K. 2000. Role of redox-regulated transcription factors in inflammation, aging and age-related diseases. *Exp. Gerontol.* **35:**521–532

Lindeman, R.D. 1990. Overview: Renal physiology and pathophysiology of aging. *Am. J. Kidney Dis.* **16:**275–282

Malmstrom, K., Murer, H. 1986. Parathyroid hormone inhibits phosphate transport in OK cells but not in LLC-PK1 and JTC-12.P3 cells. *Am. J. Physiol.* **251:**C23–C31

Mancini, M., Ricci, A., Zaccheo, D., Amenta, F. 1991. Similarity of age-dependent changes in renal and striatal dopamine receptors. *Funct. Neurol.* **6:**227–229

Manunta, P., Messaggio, E., Ballabeni, C., Sciarrone, M.T., Lanzani, C., Ferrandi, M., Hamlyn, J.M., Cusi, D., Galletti, F., Bianchi, G. 2001. Plasma ouabain-like factor during acute and chronic changes in sodium balance in essential hypertension. *Hypertension* **38:**198–203

Manunta, P., Rogowski, A.C., Hamilton, B.P., Hamlyn, J.M. 1994. Ouabain-induced hypertension in the rat: Relationships among plasma and tissue ouabain and blood pressure. *J. Hypertens.* **12:**549–560

Mauerer, U.R., Boulpaep, E.L., Segal, A.S. 1998. Regulation of an inwardly rectifying ATP-sensitive K^+ channel in the basolateral membrane of renal proximal tubule. *J. Gen. Physiol.* **111:**161–180

Meister, B., Bean, A.J., Aperia, A. 1993. Catechol-*O*-methyltransferase mRNA in the kidney and its appearance during ontogeny. *Kidney Int.* **44:**726–733

Meister, B., Fried, G., Holgert, H., Aperia, A., Hokfelt, T. 1992. Ontogeny of aromatic L-amino acid decarboxylase-containing tubule cells in rat kidney. *Kidney Int.* **42:**617–623

Mohanty, J.G., Jaffe, J.S., Schulman, E.S., Raible, D.G. 1997. A highly sensitive fluorescent micro-assay of H_2O_2 release from activated human leukocytes using a dihydroxyphenoxazine derivative. *J. Immunol. Methods* **202:**133–141

Moon, S.K., Cha, B.Y., Kim, C.H. 2003. In vitro cellular aging is associated with enhanced proliferative capacity, G_1 cell cycle modulation, and matrix metalloproteinase-9 regulation in mouse aortic smooth muscle cells. *Arch. Biochem. Biophys.* **418:**39–48

Muto, S., Asano, Y., Wang, W., Seldin, D., Giebisch, G. 2003. Activity of the basolateral K^+ channels is coupled to the Na^+/K^+-ATPase in the cortical collecting duct. *Am. J. Physiol.* **285:**F945–F954

Noulin, J.F., Brochiero, E., Coady, M.J., Laprade, R., Lapointe, J.Y. 2001. Molecular identity and regulation of renal potassium channels. *Jpn. J. Physiol.* **51:**631–647

Pedemonte, C.H., Pressley, T.A., Lokhandwala, M.F., Cinelli, A.R. 1997. Regulation of Na,K-ATPase transport activity by protein kinase C. *J. Membr. Biol.* **155:**219–227

Pedrosa, R., Soares-da-Silva, P. 2002. Oxidative and non-oxidative mechanisms of neuronal cell death and apoptosis by L-3,4–dihydroxyphenylalanine (L-DOPA) and dopamine. *Br. J. Pharmacol.* **137:**1305–1313

Pierre, S.V., Duran, M.J., Carr, D.L., Pressley, T.A. 2002. Structure/function analysis of Na^+/K^+-ATPase central isoform-specific region: Involvement in PKC regulation. *Am. J. Physiol.* **283:**F1066–F1074

Porter, N.M., Thibault, O., Thibault, V., Chen, K.C., Landfield, P.W. 1997. Calcium channel density and hippocampal cell death with age in long-term culture. *J. Neurosci.* **17:**5629–5639

Soares-da-Silva, P., Fernandes, M.H. 1991. A study on the renal synthesis of dopamine in aged rats. *Acta Physiol. Scand.* **143:**287–293

Teitelbaum, A.P., Nissenson, R.A., Zitzner, L.A., Simon, K. 1986. Dual regulation of PTH-stimulated adenylate cyclase activity by GTP. *Am. J. Physiol.* **251:**F858–F864

Tenore, G., Barili, P., Sabbatini, M., Tayebati, S.K., Amenta, F. 1997. Postnatal development of dopamine D1-like and D2-like receptors in the rat kidney: A radioligand binding study. *Mech. Ageing Dev.* **95:**1–11

Tian, G., Dang, C., Lu, Z. 2001. The change and significance of the Na^+/K^+-ATPase alpha-subunit in ouabain-hypertensive rats. *Hypertens. Res.* **24:**729–734

Vakkuri, O., Arnason, S.S., Joensuu, P., Jalonen, J., Vuolteenaho, O., Leppaluoto, J. 2001. Radioiodinated tyrosyl-ouabain and measurement of a circulating ouabain-like compound. *Clin. Chem.* **47:**95–101

Warth, R. 2003. Potassium channels in epithelial transport. *Pfluegers Arch.* **446:**505–513

Xie, Z., Askari, A. 2002. Na^+/K^+-ATPase as a signal transducer. *Eur. J. Biochem.* **269:**2434–2439

Xiong, J., Camello, P.J., Verkhratsky, A., Toescu, E.C. 2004. Mitochondrial polarisation status and $[Ca^{2+}]_i$ signalling in rat cerebellar granule neurones aged in vitro. *Neurobiol. Aging* **25:**349–359

Yu, H., Cook, T.J., Sinko, P.J. 1997. Evidence for diminished functional expression of intestinal transporters in Caco-2 cell monolayers at high passages. *Pharm. Res.* **14:**757–762

Zhou, X., Yin, W., Doi, S.Q., Robinson, S.W., Takeyasu, K., Fan, X. 2003. Stimulation of Na,K-ATPase by low potassium requires reactive oxygen species. *Am. J. Physiol.* **285:**C319–C326