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ORIGINAL PAPER

Death of ouabain-treated renal epithelial cells: evidence for p38 MAPK-mediated Na_i^+/K_i^+-independent signaling

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Abstract Recent studies demonstrate that cytotoxic actions of ouabain and other cardiotonic steroids (CTS) on renal epithelial cells (REC) are triggered by their interaction with the Na^+,K^+-ATPase α-subunit but not the result of inhibition of Na^+,K^+-ATPase-mediated ion fluxes and inversion of the $[Na^+]_i/[K^+]_i$ ratio. This study examined the role of mitogen-activated protein kinases (MAPK) in the death of ouabain-treated REC. Exposure of C7-MDCK cells that resembled principal cells from canine kidney to 3 μM ouabain led to phosphorylation of p38 without significant impact on phosphorylation of ERK and JNK MAPK. Maximal increment of p38 phosphorylation was observed at 4 h followed by cell death at 12 h of ouabain addition. In contrast to ouabain, neither cell death nor p38 MAPK phosphorylation were affected by elevation of the $[Na^+]_i/[K^+]_i$ ratio triggered by Na^+,K^+-ATPase inhibition in K^+-free medium. p38 phosphorylation was noted in all other cell types exhibiting death in the presence of ouabain, such as intercalated cells from canine kidney and human colon rectal carcinoma cells. We did not observe any action of ouabain on p38 phosphorylation in ouabain-resistant smooth muscle cells from rat aorta and endothelial cells from human umbilical vein. Both p38 phosphorylation and death of ouabain-treated C7-MDCK cells were suppressed by p38 inhibitor SB 202190 but were resistant to its inactive analogue SB 202474. Our results demonstrate that death of CTS-treated REC is triggered by Na_i^+,K_i^+—independent activation of p38 MAPK.

Keywords Na^+,K^+-ATPase · Intracellular Na^+ and K^+ · Ouabain · Cell death · p38 MAPK

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Introduction

Na^+,K^+-ATPase, a heterodimer consisting of catalytic α- and regulatory β-subunits, plays a key role in the maintenance of electrochemical gradients of monovalent cations across the plasma membrane in all types of nucleated animal cells studied so far. Numerous studies demonstrate that ouabain and other cardiotonic steroids (CTS) abolished transmembrane gradients of Na^+ and K^+ via inhibition of catalytic α1–α4 isoforms which, in turn, led to modulation of diverse Na_i^+,K_i^+—dependent cell functions such as electrical membrane potential, cell volume, transepithelial movement of salt and osmotically-obliged water, symport of Na^+ with glucose, amino acids, nucleotides, etc. [1].

In contrast to the universal impact on Na_i^+,K_i^+—dependent cell functions, long-term treatment with CTS affects cell survival in a tissue-specific manner. Thus, 48 h treatment with CTS at concentrations up to 5 mM had no impact on the survival of Rhesus monkey renal epithelial cells [2, 3], human lymphocytes [4], rat aorta smooth muscle cells [5, 6] and rat astrocytes [7], but resulted in massive

death of renal epithelial cells (REC) from the Madin–Darby canine kidney (MDCK) [8, 9]. Surprisingly, we found that in contrast to CTS, 24 h inhibition of Na^+,K^+-ATPase in K^+-free medium did not affect survival of MDCK cells [10]. This intriguing observation was subsequently confirmed by Contreras and co-workers [11]. Importantly, similarly to Na^+/K^+ pump inhibition, $[K^+]_o$ attenuation sharply increased the efficacy of CTS in triggering the cell-death machinery [10, 12]. Viewed collectively, these data strongly suggest that the cytotoxic effect of CTS, at least in these cells, is caused by their interaction with the Na^+, K^+-ATPase α-subunit but is not directly linked to elevation of the $[Na^+]_i/[K^+]_i$ ratio.

Other evidence of Na_i^+,K_i^+—independent signaling was obtained in studies of myocytes, renal epithelial and vascular endothelial cells. Exposure of these cells to low doses of CTS led to caveolin-mediated interaction of α-Na^+, K^+-ATPase with the membrane-associated nonreceptor tyrosine kinase Src and activation of diverse signaling pathways, including extracellular responsive (ERK) mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), PI3K-dependent protein kinase B (PKB), phospholipase C (PLC), $[Ca^{2+}]_i$ oscillations and increased production of reactive oxygen species (ROS), without any significant inhibition of the rate of K^+ (^{86}Rb) influx and the $[Na^+]_i/[K^+]_i$ ratio (for review, see [13–15]). In a previous investigation, we failed to detect any involvement of PLC, Ca_i^{2+} and ROS in the death of ouabain-treated MDCK cells [16]. Based on these negative results, we focused on the role of mitogen-activated protein kinases (MAPK) whose tissue-specific impact on cell survival has been shown in numerous studies [17–19]. Our data demonstrate that the death of ouabain-treated REC is mediated by activation of p38 MAPK via the Na_i^+,K_i^+—independent pathway.

Methods

Cell culture C7- and C11-MDCK cells resembling principal and intercalated cells from collecting ducts, respectively, and vascular smooth muscle cells from rat aorta (rVSMC) were isolated and maintained in culture as described previously [20]. Human umbilical vein endothelial cells (HUVEC), purchased from Clonetics (Cambrex, Walkersville, MD; cat # CC-2519), were cultured between 4 and 10 passages according to the manufacturer's instructions using endothelial growth medium (EGM-2, cat. # CC-3162). NIH 3T3 fibroblasts were purchased from the American Type Culture Collection (Rockville, MA) and cultured as described elsewhere [21]. Caco-2 cells isolated from human colon rectal carcinoma were obtained from the American Type Culture Collection and maintained in culture in $DMEM^+$ with 10% FBS. In an overwhelming number of experiments, cells were washed with phosphate-buffered saline (PBS) and incubated with or without ouabain in DMEM-like medium containing (mM) NaCl—109.4; KCl—5.4; $CaCl_2$—1.8; $MgSO_4$—0.8; $NaHCO_3$—29.8; NaH_2PO_4—0.9; HEPES—8.4; glucose—5, and vitamins and amino acids at concentrations indicated for DMEM recipes. In K^+-free medium, KCl was substituted with an equimolar amount of NaCl. Cell morphology was evaluated by phase-contrast microscopy at ×100 magnification without preliminary fixation.

Cell attachment, chromatin cleavage and caspase-3 assays were utilized to estimate the death of ouabain-treated cells. To quantify cell detachment, cells were grown in 24-well plates in the absence or presence of ouabain and the protein content of cells attached to plastic supports after three washes with 2 ml aliquots of medium W containing 100 mM $MgCl_2$ and 10 mM HEPES-Tris buffer (pH 7.4) were measured by the modified Lowry method [22]. Chromatin cleavage assay was performed as described previously [5]. Shortly thereafter, the cells grown in 24-well plates were supplied with DMEM containing serum and 0.2–0.5 μCi/ml [^{3}H]-thymidine. After 24 h, they were washed twice with 2 ml of DMEM and incubated for 24 h in isotope-free, serum-containing DMEM. Next, they were washed with PBS and incubated in 0.5 ml of isotope-free medium of different compositions for the next 18–24 h. To measure chromatin fragmentation, the plates were transferred on ice, and 1 ml of ice-cold lysed buffer (10 mM EDTA, 10 mM Tris–HCl, 0.5% Triton X-100, pH 8.0) was added. After 15 min, the cell lysate was collected, centrifuged (12,000 rpm, 10 min) and transferred for the measurement of radioactivity in a liquid scintillation spectrometer (fraction F_1). The remaining radioactivity from the sediments and wells was extracted with a 1% SDS/4 mM EDTA mixture (fraction F_2). The relative content of intracellular chromatin fragments was quantified as the percentage of total [^{3}H]-labelled DNA: $F_1/(F_1 + F_2)^{-1} \times 100\%$. Caspase-3 activity was determined as the rate of the caspase-3 inhibitor (Ac-DEVD-CHO)-sensitive component of caspase-3 fluorescent substrate (DEVD-AMC, *N*-acetyl-Asp-Glu-Val-Asp-AMC) hydrolysis according to a previously-described protocol [23].

Intracellular content of exchangeable Na^+ was measured as the steady-state distribution of extra- and intracellular ^{22}Na [5]. Briefly, cells were incubated for 8 h in DMEM or DMEM-like medium plus 2 μCi/ml $^{22}NaCl$ and ouabain as indicated in the figure legends. At the end of incubation, cells were transferred on ice, washed four times with 2 ml of ice-cold medium W containing 100 mM $MgCl_2$ and 10 mM HEPES-Tris (pH 7.4), and lysed with SDS/EDTA mixture. Radioactivity of the incubation medium and cell lysate was measured, and intracellular cations quantified as *A/am*, where *A* was the radioactivity

of the samples (cpm), *a* the specific radioactivity of Na^+ in the medium (cpm/nmol), and *m* was protein content (mg).

Western blot analysis After stimulation with the desired agonists in 6-well plates, cells were lysed on ice in 0.250 ml of buffer containing 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM EDTA, 2 mM EGTA, 25 mM HEPES (pH 7.5), 10% glycerol, 1 mM NaF, 200 μM Na_3VO_4, and protease inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM PMSF). The lysates were cleared of insoluble material by centrifugation at 20,000*g* for 10 min, treated for 5 min at 95°C and subjected to SDS–polyacrylamide gel electrophoresis using 4 and 10% polyacrylamide in stacking and resolving gels, respectively. Proteins were transferred to Hybond-C Extra nitrocellulose membranes (Amersham Biosciences, Mississauga, ON, Canada), blocked for 1 h at room temperature with 5% dry fat-free milk dissolved in TBS and incubated overnight with primary antibodies at 4°C. Subsequently, the membranes were treated with horseradish peroxide-conjugated secondary antibodies and developed by ECL Western blotting detection reagents (Amersham) in accordance with the manufacturer's instructions.

Chemicals Methyl-[^{3}H]-thymidine and ^{22}NaCl were purchased from Amersham (Mississauga, ON). DEVD-AMC, DEVD-CHO and z-VAD.fmk were obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). Antibodies for the phospho- and total-p38 MAPK and phospho-JNK MAPK were obtained from Cell Signaling (Hornby, ON). ERK antibody was kindly provided by Dr. Dulin (University of Chicago, IL). SB202190, SB202474, SP600125 and PD98059 were purchased from Calbiochem, Sigma (St. Louis, MO). The remaining chemicals were purchased from Gibco BRL (Gaithersburg, MO), Calbiochem, Sigma (St. Louis, MO) and Anachemia (Montreal, QC).

Results

Effect of ouabain and K^+-free medium on C7-MDCK cell death

Figure 1 shows that the death of ouabain-treated C7-MDCK cells occurred after 6 h reversible lag-phase with significant accumulation of dead detached cells in 8–12 h of ouabain addition. Similarly to ouabain, 6 h inhibition of Na^+,K^+-ATPase in K^+-free medium sharply increased Na_i^+ content in C7-MDCK cells (Table 1). However, in contrast to ouabain, 24 h incubation in K^+-free medium did not affect cell survival, as indicated by phase-contrast microscopy (Fig. 2a), cell attachment, chromatin cleavage and caspase-3 assays (Table 1). Consequently, 6 h treatment with ouabain was selected to search for phosphoproteins that might be involved in CTS-induced cell death

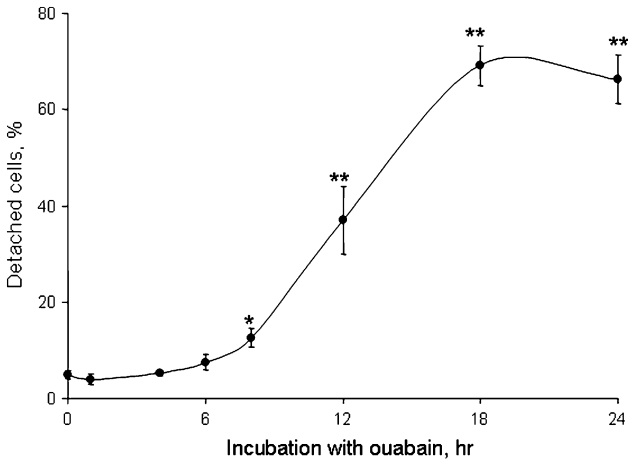

Fig. 1 Kinetics of detachment of C7-MDCK cells triggered by ouabain. Cells were treated with DMEM containing 3 μM ouabain for the time periods indicated in the *X-axis* and then transferred to ouabain-free medium to achieve an overall incubation period of 24 h. Total protein content in attached and detached cells was taken as 100%. Means ± S.E. from experiments performed in quadruplicate are shown. *, ** $P < 0.05$ and 0.001 compared to ouabain-untreated cells

signaling. K^+-free medium was employed to dissect relative impact of Na_i^+,K_i^+-mediated and -independent signaling triggered by Na^+,K^+-ATPase inhibition.

Figure 2b demonstrates that 6 h treatment with ouabain led to phosphorylation of p38 MAPK in C7-MDCK cells. Importantly, in contrast to ouabain, 6 h inhibition of Na^+,K^+-ATPase in K^+-free medium did not affect p38 phosphorylation. Electrophoretic mobility shift assay documented no action of ouabain on ERK1 and minor activation of ERK2 MAPK (Fig. 2b). Neither ouabain nor K^+-free medium altered phosphorylation of JNK1/2 MAPK (Fig. 2b) and total content of ERK, JNK and p38 MAPK (data not shown).

To further examine the role of p38 MAPK activation in the death of CTS-treated cells, we studied kinetics and dose-dependence of ouabain action on p38 phosphorylation. Figure 3a displays that p38 phosphorylation was not significantly affected during initial 2 h but was sharply increased at 4 h of ouabain addition. The delayed response was in contrast with rapid phosphorylation of ERK MAPK in rat REC [24], human and rat smooth muscles [25, 26] and rat cardiomyocytes [27]. In these cells, maximal increment of ERK phosphorylation was documented at 1–10 min of ouabain addition and was completely normalized after 1–2 h. In control medium containing 5 mM K^+, the maximal action of ouabain on p38 phosphorylation was detected at concentration of 3 μM (Fig. 3b). Transfer of C7-MDCK cells in K^+-free medium resulted in left-hand shift of dose dependency and decreased EC_{50} values for ouabain from ~0.7 to 0.1 μM (Fig. 3c).

Table 1 Effect of ouabain and K^+-free medium on Na^+ content, attachment, chromatin cleavage and caspase-3 activity in C7-MDCK cells

Parameters	Incubation medium			
	Control	Ouabain	K^+-free	K^+-free + ouabain
Intracellular Na^+ content, nmol (mg prot)$^{-1}$	52 ± 9	891 ± 67	769 ± 72	907 ± 54
Content of detached cells (%)	9.9 ± 3.3	66.1 ± 7.0	6.7 ± 2.5	65.0 ± 5.7
Chromatin cleavage (%)	4.0 ± 1.3	17.9 ± 2.61	4.6 ± 0.9	21.6 ± 2.0
Caspase-3, nmol (mg prot)$^{-1}$ h^{-1}	5.3 ± 1.8	34.1 ± 0.9	4.7 ± 1.8	36.0 ± 3.0

To measure Na_i^+ content, cells were incubated in control or K^+-free DMEM-like media ± 3 μM ouabain for 6 h; for the remaining experiments, the incubation time was extended to 24 h. For complete medium composition, see the "Methods" section. Total protein content in attached and detached cells and total [^{3}H]-labeled DNA content in were taken as 100%. Means ± S.E. from experiments performed in triplicate are shown

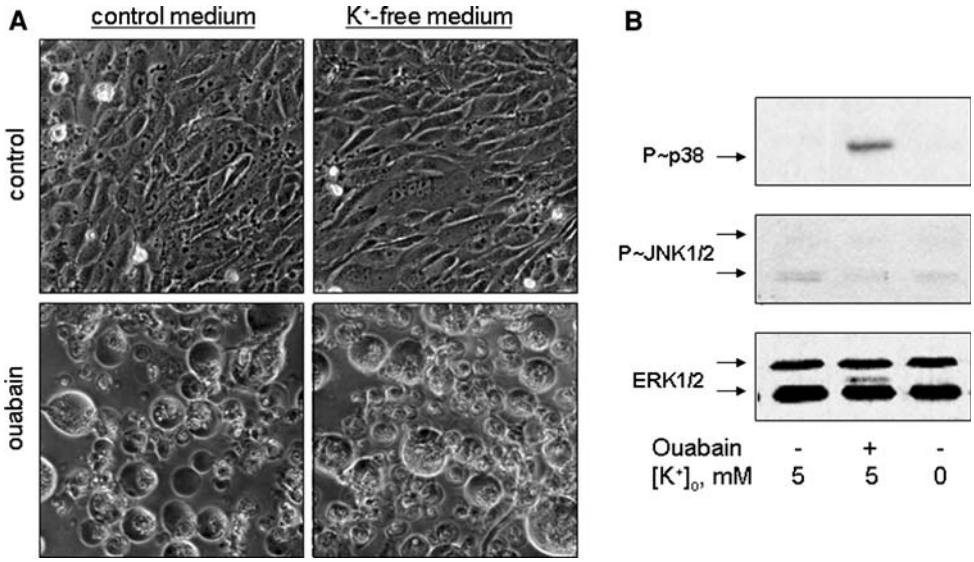

Fig. 2 Effect of ouabain and K^+-free medium on morphology (**a**) and MAPK phosphorylation (**b**) in C7-MDCK cells. **a**. Phase-contrast microscopy at 200-fold magnification after 24 h incubation of cells in control and K^+-free DMEM-like medium ± 3 μM ouabain. **b**. Representative blots demonstrating the effect of 6 h incubation with 3 μM ouabain or in K^+-free medium on the content of phosphorylated p38, JNK 1/2 and ERK 1/2 MAPK. For complete medium composition, see the Methods section

Effect of ouabain on p38 MAPK in CTS-sensitive and -resistant cell lines

Previous studies have documented that the effect of CTS on cell survival is cell-type specific. Thus, side-by-side with C7-MDCK cells resembling principal cells from canine kidney collecting ducts, long-term exposure to ouabain led to massive death of intercalated-like C11-MDCK cells [10] and Caco-2 cells isolated from human colon rectal carcinoma [13]. In contrast, 48 h exposure to high doses of ouabain did not decrease survival of rVSMC [6] and NIH 3T3 mouse fibroblasts [13]. Here, we confirm these observations by chromatin cleavage assay in cells treated with ouabain for 24 h (Table 2). Table 2 also shows that side-by-side with rVSMC and NIH 3T3 mouse fibroblasts, 24 h incubation with 3 μM ouabain did not affect chromatin cleavage in endothelial cells from human umbilical vein (HUVEC). Figure 4 displays that ouabain sharply increased p38 MAPK phosphorylation in all type of CTS-sensitive cells studied to date but did not affect its phosphorylation in CTS-resistant cells.

Effect of MAPK inhibitors

It has been demonstrated that 10 μM SB 202190 completely blocked the activity of purified p38-α and p38-β and slightly affected two other isoforms of this MAPK (p38-γ and p38-δ) [28]. Figure 5 illustrates that SB 202190 but not its inactive analogue SB 202474 protected C7-MDCK cells from the cytotoxic action of 24 h exposure to ouabain documented by phase contrast microscopy and by attenuated increments of cell detachment, chromatin fragmentation and caspase-3 activity. We did not observe any protection against cell death triggered by ouabain in the presence of SP600125 and PD98059, i.e., potent inhibitors of JNK and ERK MAPK, respectively (Table 3).

Discussion

We report that treatment with ouabain of principal and intercalated cells from collecting ducts of canine kidney (C7- and C11-MDCK, respectively) and human colon

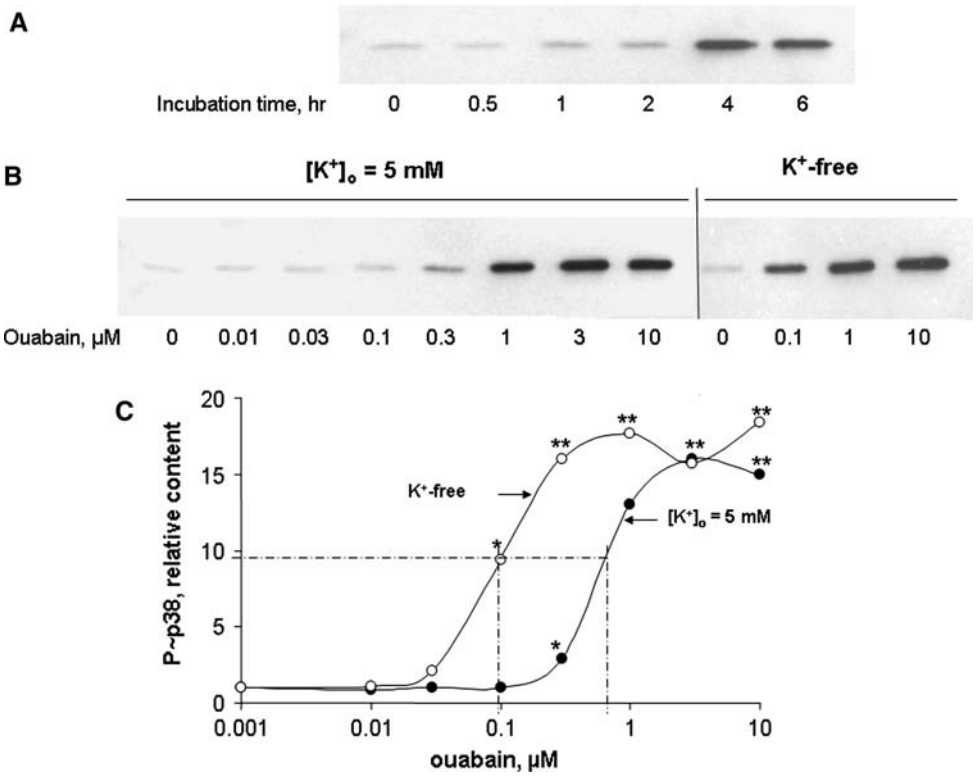

Fig. 3 Time- (**a**) and dose-dependent (**b**, **c**) actions of ouabain on p38 MAPK phosphorylation in C7-MDCK cells. **a**. Representative blots demonstrating kinetics of p38 MAPK phosphorylation in control DMEM-like medium by 3 μM ouabain. **b**. Representative blot showing dose-dependent effect of ouabain on p38 MAPK phosphorylation in 6 h of incubation in control and K^+-free medium. For complete medium composition, see the "Methods" section. **c**. Dose dependence of ouabain action on p38 MAPK phosphorylation. Optical density of phosphor-p38 bands in the absence of ouabain was taken as 1.0. Mean values from 3 experiments are shown. *, ** $P < 0.05$ and 0.01 compared to ouabain-untreated cells

Table 2 Effect of ouabain on chromatin cleavage in renal epithelial cells, vascular endothelial cells, vascular smooth muscle cells and fibroblasts

Cell types	Chromatin cleavage (%)	
	Control	Ouabain
C7-MDCK	4.8 ± 1.7	22.3 ± 5.4*
C11-MDCK	3.8 ± 2.0	29.6 ± 4.1*
CaCo-2	2.1 ± 0.8	15.4 ± 2.5*
rVSMC	3.3 ± 1.6	3.0 ± 1.8
HUVEC	4.4 ± 2.3	5.9 ± 3.0
NIH 3T3	3.1 ± 1.4	3.7 ± 1.7

Cells were treated in DMEM ± ouabain for 24 h. Ouabain was added at concentration of 3 μM (C7-MDCK, C11-MDCK, CaCo-2, and HUVEC) or 3 mM (rVSMC, NIH 3T3). Means ± S.E. from experiments performed in triplicate are shown

* $P < 0.05$ compared to controls

rectal carcinoma CaCo-2 cells leads to phosphorylation of p38 MAPK. Our results strongly suggest that activation of this signaling cascade contributes to a Na_i^+,K_i^+—independent mechanism of cytotoxic action of CTS documented in our previous studies [10, 12]. This conclusion is supported by several observations.

First, p38 phosphorylation was detected in all type of cells undergoing cell death in the presence of ouabain

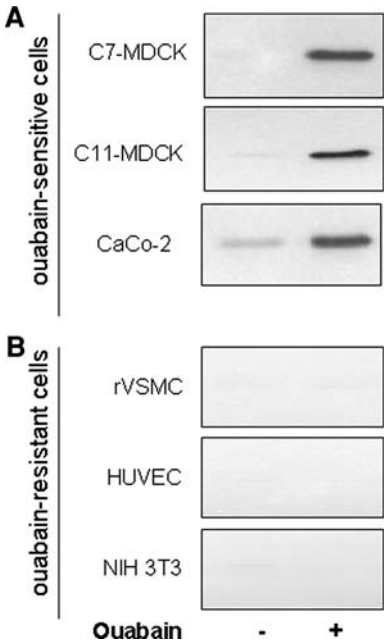

Fig. 4 Representative blots demonstrating effect of ouabain on phosphorylation of p38 MAPK in ouabain-sensitive (**a**) and -resistant (**b**) cells. Cells were treated for 6 h with 1 μM (C7-MDCK, C11-MDCK, Caco-2, HUVEC) or with 3 mM ouabain (rVSMC and NIH 3T3). Then, cell lysates were analyzed by Western blot with anti-phosho-p38 MAPK antibodies

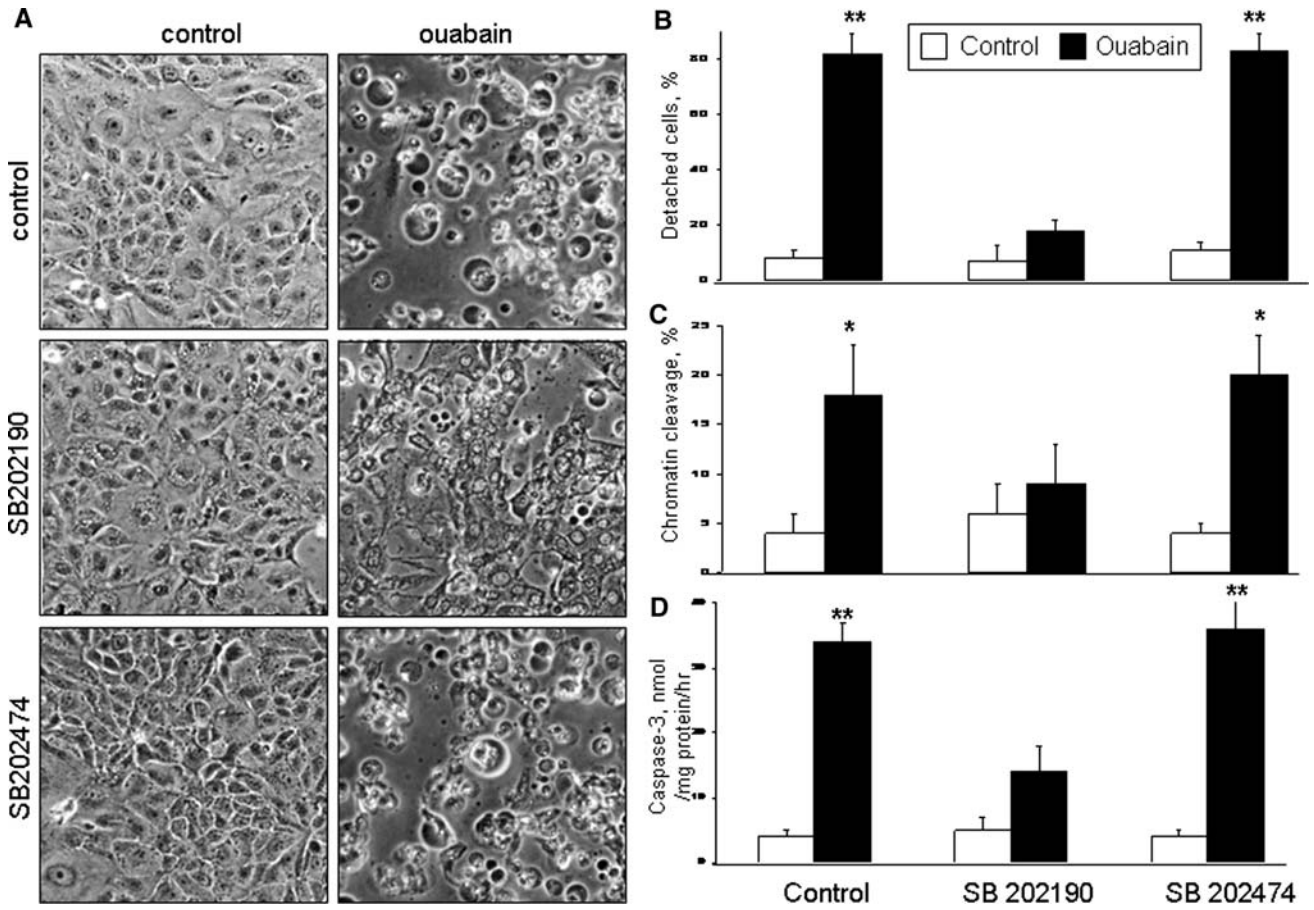

Fig. 5 Effect of ouabain on morphology (**a**), attachment (**b**), chromatin cleavage (**c**), caspase-3 activity (**d**) in C7-MDCK cells in the absence or presence of SB 202190 and SB 202474. Cells were treated in DMEM-like medium ± 3 μM ouabain, 20 μM SB 202190 or 20 μM SB 202474 for 24 h. Total contents of cells (**b**) and [^{3}H]-thymidine-labeled DNA (**c**) were taken as 100%. Phase-contrast microscopy was performed at 100-fold magnification. Means ± S.E. from 3 experiments performed in triplicate (**d**) or quadruplicate (**b**, **c**) are shown. *, ** $P < 0.05$ and 0.005 compared to ouabain-untreated cells

Table 3 Effect of JNK and ERK MAPK inhibitors on detachment of C7-MDCK cells triggered by ouabain

Additions, μM	Content of detached cells (%)	
	Without ouabain	In the presence of ouabain
None (control)	7 ± 2	85 ± 4
SP 600125, 20	10 ± 3	84 ± 7
PD 98059, 20	8 ± 4	79 ± 9

C7-MDCK cells were treated in DMEM ± 3 μM ouabain and compounds indicated in the left column for 24 h. Total protein content in attached and detached cells was taken as 100%. Means ± S.E. from experiments performed in quadruplicate are shown

(C7-MDCK, C11-MDCK CaCo-2 cells) but was absent in ouabain-resistant cells (rVSMC, HUVEC, NIH 3T3; Table 2; Fig. 4).

Second, p38 phosphorylation preceded triggering of the cell-death machinery. Indeed, p38 phosphorylation was detected at 4 h of ouabain addition (Fig. 3a) whereas the death of C7-MDCK cells was observed after a 6 h lag-phase (Fig. 1).

Third, the death of ouabain-treated C7-MDCK cells was sharply suppressed in the presence of a potent p38 MAPK inhibitor SB 202190 but not its inactive analogue SB 202474 (Fig. 5) as well as in the presence of JNK and ERK MAPK inhibitors (compounds SP600125 and PD98059, respectively; Table 3).

Fourth, in contrast to ouabain, near complete inhibition of Na^+,K^+-ATPase [10] and sharp elevation of intracellular Na^+-content triggered by 6 h incubation in K^+-free medium (Table 1) did not affect p38 phosphorylation (Fig. 2b) and survival of C7-MDCK cells (Fig. 2; Table 1). Previously, we reported that attenuation of extracellular K^+ increases efficacy of ouabain and other CTS in the triggering of the cell-death machinery [10, 12]. The same left-hand shift was observed under analysis of dose-dependent action of ouabain in control and K^+-free medium on p38 phosphorylation (Fig. 3b, c). Viewed collectively, these data strongly suggest that both p38 phosphorylation and the

death of REC are triggered by interaction of ouabain with Na^+,K^+-ATPase α-subunit but are not directly caused by inhibition of this enzyme activity and elevation of the $[Na^+]_i/[K^+]_i$ ratio.

Many studies support the tissue-specific impact of p38 in regulation of cell proliferation and death [17, 18, 29–31]. Thus, Alvardo-Kristensson and co-authors reported that p38 MAPK contributes to survival of human neutrophils by consequent phosphorylation and inhibition of caspase-8 and caspase-3 [32]. Valente and co-workers demonstrated that ouabain augments SB 202109-sensitive p38 phosphorylation in human peripheral blood mononuclear cells without any impact on cell survival [33]. In contrast, mouse and human macrophages apoptosis triggered by activation of P2X receptors and ROS is mediated by p38 activation [34, 35]. Neither survival nor p38 phosphorylation were affected in murine thymocytes subjected to 18 h incubation with 0.1 μM ouabain [19]. These data are consistent with cell-type specific actions of ouabain on p38 phosphorylation and survival demonstrated in this study.

In mammalian cells, p38 phosphorylation is activated by diverse environmental stresses such as anisosmotic media, hypoxia, heat, ultraviolet radiation as well as by lipopolysaccharides, tumor necrosis factor and other cytokines and pro-inflammatory stimuli via Rac/PKB-mediated regulation of GSK-3β and downstream MAPK kinase kinases MEKK1-4, TAK1 and ASK1 and MAPK kinases MEK3-6 [36]. It has also been demonstrated that non-phosphorylated GSK-3β blocks dimerization of MEKK4, which is required for activation of MEK4 and MEK3/6 and subsequent phosphorylation of p38 MAPK, whereas phosphorylation of GSK-3β at Ser-9 residue abolishes this inhibitory pathway [37]. Additional investigation should be performed to examine relative contribution of this signaling cascade in p38 MAPK-mediated Na_i^+/K_i^+—independent tissue type-specific cell death machinery.

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