J. Membrane Biol. 139, 183–190 (1994)

The Journal of
Membrane
Biology

The Mycotoxin Ochratoxin A Deranges pH Homeostasis in Madin-Darby Canine Kidney Cells

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Received: 23 September 1993/Revised: 18 January 1994

Abstract. Ochratoxin A (OTA) is a nephrotoxin which blocks plasma membrane anion conductance in Madin-Darby canine kidney (MDCK) cells. Added to the culture medium, OTA transforms MDCK cells in a manner similar to exposure to alkaline stress. By means of video-imaging and microelectrode techniques, we investigated whether OTA (1 μmol/liter) affects intracellular pH (pH_i), Cl^- (Cl^-_i) or cell volume of MDCK cells acutely exposed to normal (pH_{norm} = 7.4) and alkaline (pH_{alk} = 7.7) conditions. At pH_{norm}, OTA increased Cl^-_i by 2.6 ± 0.4 mmol/liter (n = 14, $P < 0.05$) but had no effect on pH_i. At pH_{alk}, application of OTA increased Cl^-_i by 8.6 ± 2.6 mmol/liter (n = 10, $P < 0.05$) and raised pH_i by 0.11 ± 0.03 (n = 8, $P < 0.05$). The Cl^-/HCO_3^- exchange inhibitor DNDS (4,4′-dinitro-stilbene-2,2′-disulfonate; 10 μmol/liter) eliminated the OTA-induced changes of pH_i and Cl^-_i. OTA did not affect cell volume under both pH_{norm} and pH_{alk} conditions.

We conclude that the OTA-induced blockade of plasma membrane anion conductance increases Cl^-_i without changing cell volume. The driving force of plasma membrane Cl^-/HCO_3^- exchange dissipates, leading to a rise of pH_i when cells are exposed to an acute alkaline load. Thus, OTA interferes with pH_i and Cl^-_i homeostasis leading to morphological and functional alterations in MDCK cells.

Key words: Ochratoxin A — MDCK cells — Intracellular chloride — Intracellular pH — DNDS

Introduction

The nephrotoxin Ochratoxin A (OTA) is ubiquitously distributed in fungal-contaminated nutrients [15]. It causes severe impairment of kidney function in several species [14, 25] and increases the incidence of renal adenoma and carcinoma [1]. The impairment of the kidney to concentrate the final urine [14] suggests that OTA may act on nephron portions beyond those of the proximal tubule. In agreement with this view, in collecting duct-derived MDCK cells [27] OTA reduces the plasma membrane anion conductance at concentrations 20 times lower than those of the Cl^--channel blocker 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) [8, 29].

When OTA is added to MDCK cells in culture, phenotypical changes occur, identical to those observed in MDCK cells grown under alkaline stress [31]. To investigate whether OTA may disturb intracellular pH homeostasis via inhibition of plasma membrane anion conductance, we measured intracellular pH (pH_i) and intracellular Cl^- activity (Cl^-_i) in response to acute OTA exposure, at normal extracellular pH (pH = 7.4) and during alkalosis (pH = 7.7). Because the activity of the anion exchanger depends on pH_i [10], alkalosis was used to activate Cl^-/HCO_3^- exchange, thus unmasking a possible effect of OTA on pH_i and Cl^-_i.

We found that OTA leads to an increase of intracellular Cl^-, which could be prevented by the addition of the Cl^-/HCO_3^- exchange blocker DNDS [7]. Furthermore, OTA increased intracellular pH at conditions in which Cl^-/HCO_3^- exchange was active.

Materials and Methods

MDCK Cell Culture

Cells ($2 \cdot 10^6$) obtained at passage 53 from the American Type Culture Collection were seeded in coated plastic culture dishes (growth area = 75 cm^2; Nunc, Wiesbaden, Germany) in 10 ml Minimum Es-

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sential Medium with Earle's salts, nonessential amino acids and L-glutamine (MEM, Biochrom KG, Berlin, Germany) and cultured under standard cell culture conditions (37°C, 5% CO_2). MEM was supplemented with 10% fetal calf serum (Biochrom KG) and 26 mmol/liter $NaHCO_3$. The medium was changed three times a week and the cells were split once a week. Under control conditions, MDCK cells form confluent monolayers. For the measurement of membrane potential, intracellular Cl^- activity, intracellular pH and cell volume, MDCK cells were seeded on thin glass microscope coverslips pretreated with poly(L-)lysine (0.1 g/liter; Serva, Heidelberg, Germany).

For the investigation of cell shape and cell growth in the presence of the mycotoxin, cells were grown in a medium containing 10 μmol/liter OTA. After three days, numerous spindle-shaped cells could be detected, while the formation of a confluent monolayer was lacking. The cells were then removed by short incubation with trypsin (Biochrom KG) and transferred to a new petri dish. This procedure was repeated four times (in intervals of four days), resulting in an enrichment of spindle-shaped cells. For cloning, cells were removed from the dish, diluted and seeded in a 96-well dish. The cloned cells grown on regular culture medium were called O-MDCK cells. The same procedure applying alkaline MEM leads to similar cell transformation as reported previously [21, 31].

Electrophysiological Measurements

Single cells (MDCK cells as well as O-MDCK cells) were fused to form giant cells according to the techniques published by Kersting et al. [11]. Plasma membrane potential (PD) of O-MDCK cells was measured with electrophysiological techniques recently described in detail [31]. Cells were impaled with conventional microelectrodes filled with 1 mol/liter KCl. The microelectrodes were connected via Ag/AgCl half-cells to a high impedance electrometer (FD 223, WPI, Hamden, CT).

Measurements of Intracellular Chloride Activity

Fused MDCK cells were impaled with a liquid ion-exchange chloride-selective microelectrode (resin no. 477913, Corning Glass, Medfield, MA) and a conventional PD microelectrode. Measurements were performed as reported previously [13]. The slopes of the electrodes were between 50 and 58 mV for a 10-fold increase in Cl^- activity. Intracellular Cl^- activities (Cl^-_i) were calculated according to the equation $Cl^-_i = Cl^-_o \cdot \exp10 \cdot (PD^{Cl} - PD)/\text{slope}$, where Cl^-_o is the extracellular Cl^- concentration and PD^{Cl} the electrochemical potential difference of Cl^-.

The Ringer solution used for superfusion was composed as follows (in mmol/liter): 110 NaCl, 24 $NaHCO_3$, 5.4 KCl, 1.2 $CaCl_2$, 1 NaH_2PO_4/Na_2HPO_4, 0.8 $MgCl_2$, 5.5 D-glucose; gassed with 5% CO_2 to a pH of 7.4 at 37°C. To obtain an extracellular pH of 7.7, the Ringer solution contained 48 mmol/liter $NaHCO_3$ (gassed with 5% CO_2) and the concentration of NaCl was reduced by 24 mmol/liter.

Measurements of Intracellular pH

Intracellular pH of single cells was determined using the pH-sensitive dye BCECF (2′7′-μbis-(2-carboxyethyl)-5,6-carboxyfluorescein, Molecular Probes, Eugene, OR) as described elsewhere [5, 26, 30]. In brief, cells were incubated with MEM containing BCECF in a final concentration of 3 μmol/liter for 15 min. The coverslips then were rinsed four times with superfusion solution to remove the BCECF-containing medium. The coverslips were transferred to the stage of an inverted IM 35 Zeiss microscope (630× magnification, oil immersion). Excitation light source was a 50 W mercury lamp. The excitation wavelengths were 436 ± 5 and 485 ± 5 nm, the emitted light was filtered through a bandpass-filter (515–565 nm). The data acquisition rate was one fluorescence intensity ratio (485/436 nm) every 10 sec. Images were digitized on-line using a video-imaging software (Java, Jandel Scientific, Corte Madera, CA). After background subtraction, fluorescence intensity ratios (485/436 nm) were calculated. Calibration was performed after each experiment by the nigericin (Sigma, St. Louis, MO) technique [26, 30], using at least three calibration solutions in the range from pH 6.8 to 7.8.

Measurement of Intracellular Free Calcium (Ca^{2+}_i)

Ca^{2+}_i was determined using the Ca^{2+}-sensitive dye Fura 2-AM (Molecular Probes) as described elsewhere [3, 9, 32]. In brief, cells were incubated with MEM containing Fura 2-AM in a final concentration of 5 μmol/liter for 45 min. After rinsing, the coverslips were mounted on the stage of an inverted Axiovert 100 TV microscope (400× magnification, oil immersion; Zeiss, Oberkochen, Germany). The fluorescence signal was monitored at 500 nm with excitation wavelength alternating between 334 and 380 nm using a 100 W Xenon lamp and an automatic filter change device (Zeiss). Filter change and data acquisition were controlled by the Attofluor™ software (Zeiss). $[Ca^{2+}]_i$ was calculated according to the method of Grynkiewicz et al. [9] with a dissociation constant of 225 nmol/liter. The maximum and minimum fluorescence ratios (R_{max} and R_{min}) were measured after addition of the calibration solutions. R_{max} was measured in the presence of Ringer containing 3 mmol/liter Ca^{2+} and 1 μmol/liter ionomycin (Sigma), R_{min} was measured in the presence of Ca^{2+}-free Ringer solution containing 10 mmol/liter EGTA and 1 μmol/liter ionomycin.

Monitoring of Cell Volume

Cell volume was monitored with the above-described microscope and video-imaging software. As described by Kersting et al. [13], relative changes of cell diameter are in the same range as relative changes of cell volume. Thus, we determined two perpendicular diameters for each cell at the different times of the measurement and related the mean of them to the mean of the diameters at time = 0 min (=100%).

Chemicals

Ochratoxin A was obtained from Sigma. DNDS was a gift of Dr. Frölich, Chicago, IL. All other substances were of analytical grade (purchased from Merck, Darmstadt, FRG), if not stated otherwise.

Statistics

The data are presented as mean values ± SEM. Significance of difference was tested by paired or unpaired *t*-test as applicable. Differences were considered significant if $P < 0.05$.

Results

Effect of OTA on the Phenotype of MDCK Cells

Exposure of MDCK cells to OTA (10 μmol/liter, n = 4) over 3–4 days led to sustained phenotypical changes identical to those maintained at alkaline stress [31]. The OTA-treated cells (O-MDCK cells) retained their

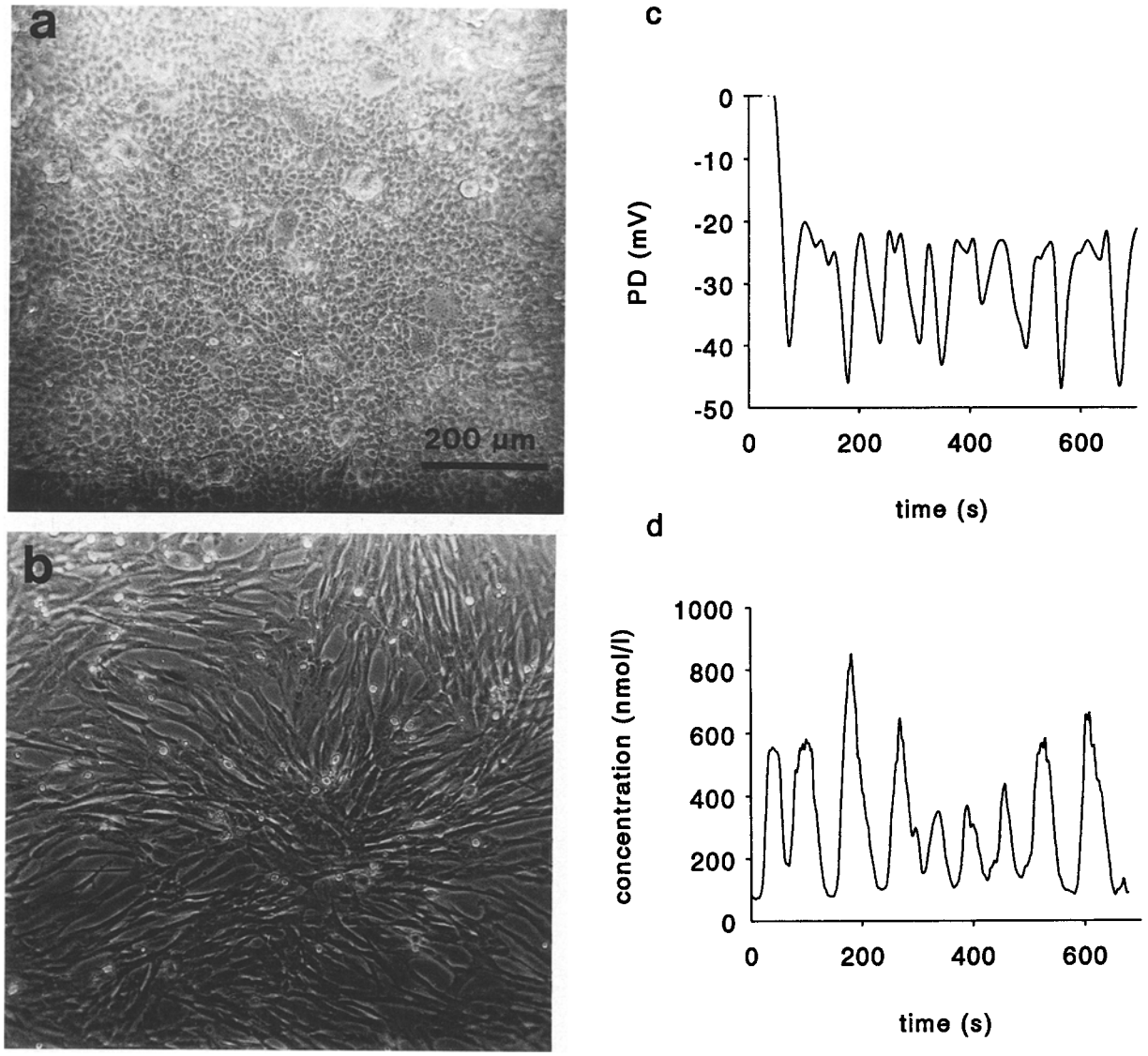

Fig. 1. (*a*) Confluent monolayer of normal wild-type MDCK cells maintained in standard culture medium. (*b*) Cloned O-MDCK cells maintained in standard culture medium for three weeks (i.e., after nine splitting procedures). The pleiomorphic cells grow irregular in multilayers and lack contact inhibition. (*c*) Plasma membrane potential (PD) recorded in a fused O-MDCK cell. PD shows spontaneous hyperpolarizing spikes. (*d*) Intracellular free Ca^{2+} concentration of O-MDCK cells exhibits spontaneous spikes, which probably trigger the hyperpolarizing spikes of PD. Representative tracing from a single cell (n = 10).

abnormal phenotypical growth pattern in regular culture medium. The O-MDCK cells are unable to form an organized tight monolayer in contrast to the normal MDCK parent cells (Fig. 1*a* and *b*). O-MDCK cells are spindle-shaped and lack contact inhibition as indicated by multilayered spots (so-called foci). The membrane potential of O-MDCK cells was not stable but showed spontaneous hyperpolarizing spikes (Fig. 1*c*) with an amplitude of 2C ± 5 mV and a frequency of 1.0 ± 0.1 spikes/min (n = 6). Furthermore, O-MDCK cells exhibited spontaneous cytosolic Ca^{2+} spikes (Fig. 1*d*) with a frequency of 0.7–1 spikes/min (n = 10), which probably trigger the spontaneous PD changes via Ca^{2+}-sensitive K^+-channels [24, 32]. The morphological and functional characteristics of O-MDCK cells are virtually identical to those of MDCK-F cells reported previously [31, 32].

Effect of OTA on Intracellular Cl^- Activity (Cl^-_i)

Cl^-_i in fused MDCK cells under control conditions (extracellular pH 7.4) was 54 ± 4 mmol/liter (n = 25; Table 1). When 1 µmol/liter OTA is added under pH control conditions, Cl^-_i increased significantly by 2.6

Table 1. PD, Cl^-_i and pH_i of fused MDCK cells at control pH (7.4) and alkaline pH (7.7) in the superfusion

	pH 7.4	pH 7.7
PD (mV)	−23.2 ± 3.7 (30)	−24.6 ± 3.6 (15)
Cl^-_i (mmol/liter)	54 ± 4 (25)	45 ± 5 (10)*
pH_i	7.19 ± 0.02 (20)	7.30 ± 0.02 (10)*

PD = plasma membrane potential. Cl^-_i = intracellular chloride activity. pH_i = intracellular pH. *$P < 0.05$ compared to control (n).

± 0.4 mmol/liter (n = 15; Fig. 2*a* and Table 2). Cl^-_i approached the plateau value within 2–4 min. Cl^-_i of fused MDCK cells under alkaline conditions (extracellular pH 7.7) was 45 ± 5 mmol/liter (n = 10; Table 1). In this case, addition of 1 µmol/liter OTA led to a significant increase of Cl^-_i of 8.6 ± 2.6 mmol/liter (n = 10; Fig. 2*b* and Table 2) within 2–4 min. The OTA-induced increase of Cl^-_i at pH 7.7 was significantly larger compared to the increase at pH 7.4. In the presence of the Cl^-/HCO_3^- exchange blocker DNDS (10 µmol/liter), Cl^-_i was 26.7 ± 1.0 mmol/liter (n = 5; Table 3). Addition of OTA in the presence of DNDS had no effect on Cl^-_i at pH 7.7 (n = 5; Table 3 and Fig. 5*a*).

Effect of OTA on Intracellular pH (pH_i)

At control conditions (pH 7.4), pH_i was 7.19 ± 0.02 (n = 20; Table 1). The addition of OTA had no effect on pH_i (Table 2). In the presence of DNDS, pH_i was 7.15 ± 0.04 (n = 7). The absence of an alkalinizing effect of DNDS indicates that Cl^-/HCO_3^- exchange is not active under control conditions. Under alkaline conditions (pH 7.7), pH_i was 7.30 ± 0.02 (n = 10; Table 1). The addition of OTA led to an alkalinization of the cells within 3–5 min. pH_i increased significantly by 0.11 ± 0.03 (n = 10; Fig. 3 and Table 2) to 7.41 ± 0.05. In the presence of DNDS (extracellular pH 7.7), pH_i was 7.45 ± 0.04 (n = 7; Table 3), indicating that Cl^-/HCO_3^- exchange is active at an alkaline pH. Furthermore, DNDS (10 µmol/liter) prevented the alkalinizing effect of OTA on pH_i (ΔpH_i = 0.001 ± 0.03, n = 7; Table 3 and Fig. 5*b*).

Effect of OTA on Cell Volume

Figure 4 shows the relative changes of cell volume under control and alkaline conditions, respectively. The mean cell diameter was 37.3 ± 1.3 µm (n = 17). When the superfusion solution was changed from control to alkaline conditions, no effect on cell volume was observed (relative cell volume under alkaline conditions

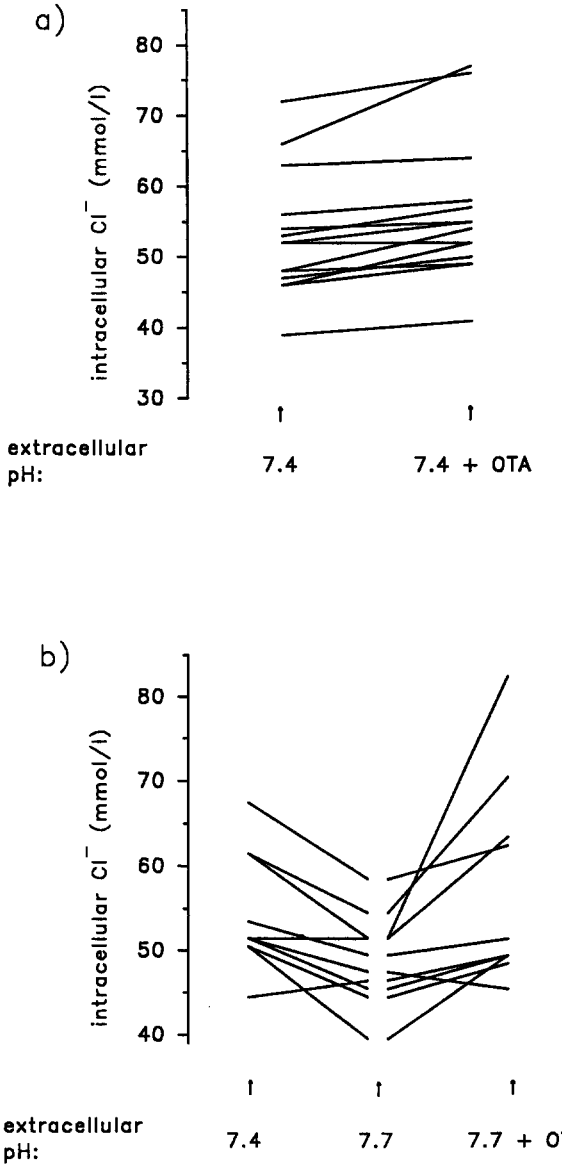

Fig. 2. (*a*) Effect of OTA (1 µmol/liter) on the intracellular Cl^- activities at normal pH (7.4). Mean Cl^-_i change is statistically significant. (*b*) Effect of OTA (1 µmol/liter) on the intracellular Cl^- activity at alkaline pH (7.7). Mean Cl^-_i change is statistically significant.

Table 2. Change of PD, Cl^-_i and pH_i of fused MDCK cells at control pH (7.4) and alkaline pH (7.7) in presence of OTA (1 µmol/liter)

	pH 7.4	pH 7.7
ΔPD (mV)	+0.6 ± 0.3 (15)	−3.1 ± 0.9 (10)[a,b]
ΔCl^-_i (mmol/liter)	+2.6 ± 0.4 (15)[a]	+8.6 ± 2.6 (10)[a,b]
ΔpH_i	−0.02 ± 0.02 (6)	+0.11 ± 0.03 (10)[a,b]

ΔPD = change of plasma membrane potential. ΔCl^-_i = change of intracellular chloride activity. ΔpH_i = change of intracellular pH. [a]Significantly different from zero. [b]$P < 0.05$ compared to the changes at control pH (7.4), (n).

Table 3. PD, Cl^-_i and pH_i of fused MDCK cells in the presence of DNDS (10 μmol/liter) at alkaline pH (7.7) and changes (Δ) induced by OTA (1 μmol/liter)

PD (mV)	−36.4 ± 2.4 (5)*	ΔPD	−0.4 ± 1.5 (5)
Cl^-_i (mmol/liter)	26.7 ± 1.0 (5)*	ΔCl^-_i	−1.6 ± 2.5 (5)*
pH_i	7.45 ± 0.04 (7)*	ΔpH_i	0.001 ± 0.03 (7)*

PD = plasma membrane potential. Cl^-_i intracellular chloride activity. pH_i = intracellular pH. DNDS = 4,4′-dinitro stilbene-2,2′-disulfonate. *$P < 0.05$ compared to experiments at pH 7.7 but in absence of DNDS, (n).

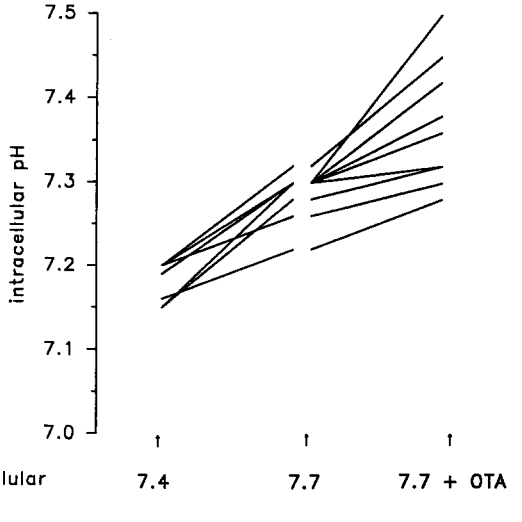

Fig. 3. Effect of OTA (1 μmol/liter) on the intracellular pH at alkaline pH (7.7). pH_i changes are statistically significant.

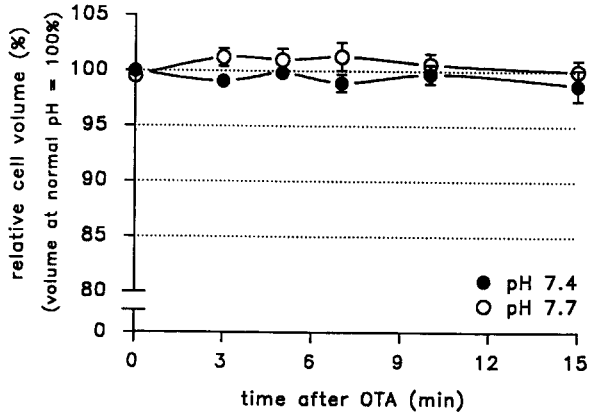

Fig. 4. Relative cell volume of fused MDCK cells after addition of OTA (1 μmol/liter) at control (n = 8) and alkaline pH (n = 9). The volume before addition of OTA at control conditions (pH 7.4) refers to 100%. Superfusion at alkaline conditions (pH 7.7) had virtually no effect on relative cell volume (99.5 ± 0.7%). Furthermore, OTA did not change the relative cell volume significantly.

was 99.5 ± 0.7%, n = 9). Neither under control (n = 8) nor alkaline conditions (n = 9) did OTA induce significant changes in relative cell volume (Fig. 4).

Discussion

The mycotoxin Ochratoxin A was first described by Merwe et al. [17] in 1965. Subsequent studies unmasked OTA as a toxin causing nephropathies [14] and kidney carcinomas [1]. Pathological findings such as polyuria and reduced urine osmolality [2, 14], drew attention to effects of OTA on nephron sections beyond the proximal tubule [8]. To study the action of OTA at the cellular level, we used Madin-Darby canine kidney (MDCK) cells, which resemble the intercalated cells of renal collecting duct [20, 27].

The function of intercalated cells strongly depends on plasma membrane Cl^- conductance [4, 22]. Chloride ions enter the cell via Cl^-/HCO_3^- exchange [6] and exit the cell through a Cl^--conductive pathway driven by an outward-directed electrochemical driving force. Inhibition of the Cl^- exit step should result in intracellular Cl^- accumulation and, thus, reduce the driving force of Cl^-/HCO_3^- exchange. Consequently, intracellular pH is expected to rise.

In a previous study [8], we showed that in MDCK cells OTA impairs plasma membrane anion conductance even more effectively than the potent Cl^--channel blocker NPPB (5-nitro-2-(3-phenylpropylamino) benzoic acid [29]). Furthermore, OTA mimics the effect of alkaline stress on MDCK cells. Exposure of MDCK cells to OTA in culture led to irreversible cell transformation (Fig. 1), similar to that observed with alkaline stress [31]. Hence, we investigated whether OTA deranges intracellular acid-base homeostasis due to its interaction with plasma membrane anion conductance. Preliminary data obtained using the patch clamp technique (inside-out patch mode) showed that application of OTA led to a decrease of the open probability of Cl^- channels in MDCK cells (*unpublished results*).

Under control conditions, intracellular Cl^- activity was significantly above the activity calculated for passive distribution of chloride across the plasma membrane (calculated concentration = 48.1 ± 6.1 mmol/liter, compare with the actual concentration of 54 ± 4 mmol/liter; Table 1). Under alkaline conditions, the dif-

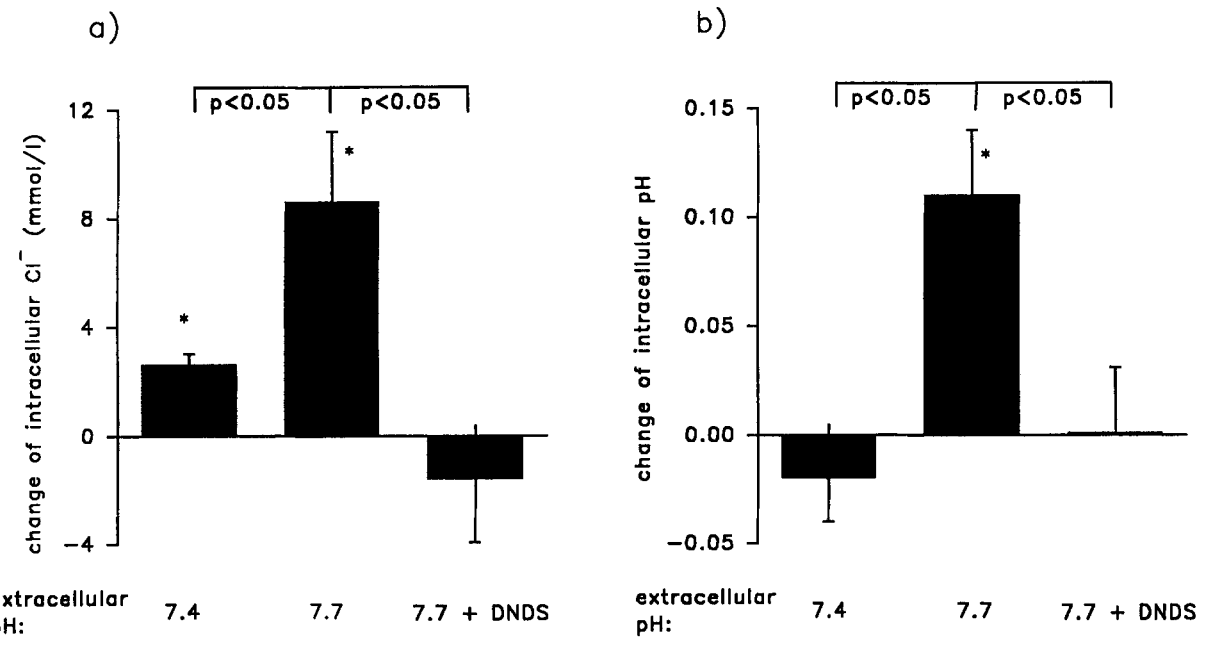

Fig. 5. (*a*) Change of intracellular Cl^- concentration after addition of OTA (1 μmol/liter) at control pH (7.4), alkaline pH (7.7) and alkaline pH in the presence of DNDS (10 μmol/liter). * Significantly different from zero. (*b*) Change of intracellular pH after addition of OTA (1 μmol/liter) at control pH (7.4), alkaline pH (7.7) and alkaline pH in the presence of DNDS (10 μmol/liter). * Significantly different from zero.

ference between concentration calculated for passive distribution (passive distribution = 36.1 ± 5.3 mmol/liter) and the intracellular Cl^- concentration measured (45 ± 5 mmol/liter; Table 1) increased about threefold. Thus, the electrochemical driving force for Cl^- fluxes was directed outwards across the plasma membrane in both cases. The addition of OTA led to an increase of Cl^-_i due to the blockade of Cl^--conductive pathway. Activation of Cl^-/HCO_3^- exchange at alkaline intracellular pH [10] resulted in an OTA-induced increase of Cl^-_i about threefold compared to the OTA-induced increase observed at control pH. In both cases, cell volume remained constant, so that the increase of Cl^-_i stands for intracellular Cl^- accumulation. In the presence of DNDS (at alkaline pH), the intracellular activity of chloride calculated for passive distribution (mean = 23.1 ± 3.2 mmol/liter) was not significantly different from the activity measured (26.7 ± 1 mmol/liter; Table 3). Since DNDS is a very potent blocker of Cl^-/HCO_3^- exchange (binding constant 90 nmol/liter for human erythrocyte anion transporter [7]), it can be assumed that Cl^-/HCO_3^- exchange was completely blocked in these experiments. Thus, the passive distribution of chloride across the plasma membrane in the presence of DNDS indicates that Cl^-/HCO_3^- exchange is virtually the only system for active Cl^- uptake at alkaline pH. Blockade of Cl^-/HCO_3^- exchange abolishes the OTA-induced Cl^- accumulation. These findings indicate that OTA impairs transmembrane recycling of Cl^-, which enters the cell by Cl^-/HCO_3^- exchange.

The increase of Cl^-_i decreases the net driving force of plasma membrane Cl^-/HCO_3^- exchange and, thus, should lead to intracellular alkalinization if Cl^-/HCO_3^- exchange is active. Addition of OTA at alkaline pH (i.e., at activated Cl^-/HCO_3^- exchange) increased intracellular pH significantly but had no significant effect when added at control pH (at inactivated Cl^-/HCO_3^- exchange). If this effect was due to the reduced driving force of Cl^-/HCO_3^- exchange, DNDS should abolish the effect of OTA. This was indeed the case. Addition of DNDS resulted in a rise of cellular pH only at alkaline pH, indicating that Cl^-/HCO_3^- exchange is active only at alkaline pH. At control pH, Cl^-/HCO_3^- exchange is inactive [10]. Therefore, Cl^- accumulation in the cells was lower and pH_i remained virtually constant. Under these conditions, transport of Cl^- into the cells is probably mediated by the Na^+-K^+-$2Cl^-$ cotransporter, known to be present in these cells [16].

Addition of OTA or DNDS, respectively, led to a hyperpolarization of PD. This hyperpolarization was most probably the result of an increase of the (relative and/or absolute) plasma membrane K^+ conductance. In the case of OTA, the decrease of the plasma membrane anion conductance known to occur at these conditions [8] may have increased the K^+ conductance relative to the overall membrane conductance. The change of PD after addition of DNDS was fourfold compared

to the PD change after addition of OTA. Most likely, this hyperpolarization was due to the pronounced DNDS-induced alkalinization known to stimulate the K^+ channels of the plasma membrane [19]. Additionally, there might be an effect of rheogenic HCO_3^- transport leading to further hyperpolarization as described for other stilbene derivates [28].

We conclude from our results that the reduction of plasma membrane anion conductance by OTA, as described in a previous study [8], deranges intracellular acid-base homeostasis in MDCK cells. The blockade of the Cl^--conductive pathway leads to intracellular accumulation of Cl^- which enters the cell through Cl^-/HCO_3^- exchange. Consequently, the driving force of the Cl^-/HCO_3^- exchanger for HCO_3^- extrusion decreases, leading to intracellular alkalinization. This action of OTA may explain the sustained morphological and functional alterations in MDCK cells. Depending on the functional state of the MDCK cells in culture [12, 23], OTA may more or less lead to intracellular alkalinization and thereby induce alkaline stress. Intracellular pH may be remarkably elevated when cells are growing [18], thus possibly representing a vulnerable phase to OTA during cell proliferation. If the observed effect of OTA on cells in culture holds true for collecting duct cells in vivo, the disturbance of cellular acid-base homeostasis could explain at least some functional alterations induced by OTA [8], such as reduced excretion of titrable acid, and thus, could be the initial event of OTA-induced nephropathy.

This study was carried out with the technical assistance of Sigrid Mildenberger and Ruth Freudinger.

The work was supported by the Deutsche Forschungsgemeinschaft (DFG, Si 170/7-1).

We thank the Zeiss Company (Oberkochen, Germany) for providing the Attofluor™ video-imaging system for the intracellular Ca^{2+} measurements.

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