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Functional Linkage between the Transport Characteristics of the MDCK1 Cell Monolayer and Their Actin Cytoskeleton Organization

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Abstract—The dynamics of the actin cytoskeleton spatial organization and transepithelial electric resistance (TEER) in the MDCK1 cell monolayer exposed to arginine–vasopressin (AVP) and forskolin, a protein kinase A (PKA) activator, have been studied. These physiologically active substances are shown to depolymerize filamentous actin in MDCK1 cells (in both the apical and basal cytoplasm) and, concurrently, to considerably decrease the TEER of the cell monolayer. A decrease in TEER suggests an increase in the ion current through the cell monolayer. Correspondingly, the created ion gradient stimulates AVP-sensitive water flow. To clarify the routes of ions and water in MDCK monolayer, the localization of claudin-1 and -2 in tight junctions of ATCC (American Type Culture Collection) MDCK (a low TEER) and MDCK1 (a high TEER) cells was studied by immunofluorescence assay. Claudin-1 and -2 are detectable in the tight junctions of ATCC MDCK cells; however, the tight junctions of MDCK1 cells contain only claudin-1, whereas pore-forming claudin-2 is absent. The exposure of MDCK1 cells to forskolin fails to change the distribution of the studied claudins, thereby suggesting that a decrease in TEER caused by forskolin is associated with a change in transcellular, rather than paracellular, permeability of the monolayer

Keywords: arginine–vasopressin, MDCK cells, transepithelial resistance, actin cytoskeleton
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INTRODUCTION

The MDCK epithelial cell line derived from the canine kidney reproduces in vitro the most important morphofunctional features of transport epithelium in collecting tubules, namely, separation of the cell membrane into apical and basolateral domains, the presence of zones of tight intercellular junctions separating the apical and basolateral membrane domains, and polarized distribution of several transport membrane proteins (Handler, 1983; Kreisberg, Wilson, 1988; Gekle et al., 1994). These specific features in the MDCK cell line has allowed for its repeated use as a model object for studying transport of various substances—in particular, ions and water—in polarized epithelia. It has been shown that MDCK cells are able to directly transfer sodium ions and water from the apical to the basolateral surface of the monolayer. According to the available data, MDCK monolayer under standard cultivation conditions transports approximately 2.6 μM Na^+/h cm^2 in the apical–basolateral direction (Cereijido et al., 1981a, 1981b; Oberleithnern, 1990). Directed sodium transport through the cell monolayer creates an ion gradient, entailing a transepithelial osmotic water flow. The ability of MDCK cells to transfer ions and water through the epithelium manifests itself in the emergence of "domes" or blisters, dome-shaped monolayer regions locally separated from the substrate and filled with fluid (Cereijido et al., 1981a; Tanner et al., 1984).

The transepithelial sodium transport is mediated by the amiloride-sensitive epithelial Na channels (ENaCs) localized to the MDCK cell apical membrane (Saier et al., 1986) and Na^+/K^+-ATPase localized to their basolateral membrane (Caplan et al., 1986; Bystriansky, Kaplan, 2007). The entry of sodium ions into cells via the ENaCs is the particular event that limits the total rate of transepithelial sodium flux; thus, amiloride-sensitive sodium channels are the target of the main cell mechanisms regulating sodium transport (Marunaka, 1997; Loffing, Korbmacher, 2009).

[1] *Abbreviations:* cAMP—cyclic adenosine monophosphate, ATCC—American Type Culture Collection, AVP—arginine vasopressin, MR—mineralocorticoid receptor, PKA—protein kinase A, ENaC—epithelial Na channel, TEER—transepithelial electric resistance.

Sodium reabsorption in the renal collecting tubules is controlled by several signaling molecules. The main regulators of this process are the hormones aldosterone (in some animals, deoxycorticosterone) and arginine vasopressin (Reif et al., 1986; Bens et al., 2006). These hormones also considerably increase sodium transport in the MDCK cell monolayer (Blazer-Yost et al., 1996).

Aldosterone binds to the mineralocorticoid receptor (MR) located on the basolateral surface of the principal cells of collecting ducts. The intracellular action of aldosterone (similar to the other steroid hormones) is associated with the MR transport to the nucleus and upregulation of several genes, in particular, the genes encoding ENaC α-subunit (Loffing et al., 2001), Na^+/K^+-ATPase (Verrey et al., 1989; Vinciguerra et al., 2005), and protein kinase SGK-1 (Chen et al., 1999). SGK-1 phosphorylates Nedd4-2 protein, thereby inactivating it. The Nedd4-2 in an active form catalyzes ENaC ubiquitination, which enhances ENaC internalization from the apical membrane to cytoplasm with further degradation. The phosphorylated Nedd4-2 loses this activity (Staub et al., 2000; Eaton et al., 2010). Thus, the upregulation of the *SGK-1* gene shifts the balance between the ENaC integration into the membrane and its internalization towards an increase in the number of ENaCs, thereby increasing sodium reabsorption.

There are several hypothesizes regarding the nongenomic cellular effects of aldosterone putatively associated with an increase in the intracellular calcium concentration and activation of protein kinase C; however, the corresponding receptor and signaling mechanisms of these effects are thus far rather vague (for a review, see Verhovez et al., 2012).

Traditionally, arginine vasopressin (AVP) is primarily regarded as an antidiuretic hormone, regulating the water reabsorption by renal collecting ducts. An antidiuretic effect of AVP is rather well studied and is associated with the AVP-induced translocation of the aquaporin-2 water channel from the pool of cytoplasmic vesicles to the apical membrane of collecting duct principal cells (Nielsen et al., 1995; Nejsum et al., 2005). The intracellular signaling that mediates this process comprises AVP binding to a V2 receptor in the basolateral membrane of epithelial cells, interaction of the receptor with the G_s protein α-subunit, activation of adenylate cyclase, cAMP synthesis, activation of protein kinase A (PKA), and phosphorylation of aquaporin-2 (Skorecki et al., 1992; Barberis et al., 1998; Ivanova, 1999; Brown et al., 2009). However, along with water transport, AVP also directly stimulates the transepithelial sodium transport in collecting ducts (Frindt, Burg, 1972; Bankir et al., 2005). Interestingly, this stimulation is implemented with involvement of the same signaling mechanisms (PKA signaling pathway) as the stimulation of osmotic water flow (Schafer, Troutman, 1990; Garty, Palmer, 1997; Morris, Schafer, 2002; Mel'nitskaya et al., 2006). According to the available data, the cellular basis for an antinatriuretic effect of AVP is the regulation of ENaC traffic to the apical membrane of collecting duct principal cells (similar to the aquaporin-2 traffic in the AVP antidiuretic effect; Erlij et al., 1999; Butterworth et al., 2005). Thus, the cellular mechanisms that regulate transepithelial water transport and transepithelial sodium transport via AVP are similar. In general, the tightest functional synergy is observed between the AVP-controlled reabsorption of sodium ions and water in the renal collecting ducts, since the transepithelial sodium flux is the main component that creates the osmotic gradient necessary for the AVP-induced water flow (Bugaj et al., 2009; Stockland, 2010).

There is convincing evidence that the function of various membrane transport proteins in epithelia is modulated by the submembrane actin cytoskeleton (for a review, see Mazzochi et al., 2006a). The idea that actin plays an important role in implementing the ENaC function is, first and foremost, based on data on the change in transmembrane sodium current induced by cytochalasin (Cantiello et al., 1991). In this work, we have demonstrated that the fragmentation of submembrane microfilament network caused by 5-min exposure to cytochalasin D considerably increases the sodium flux through the apical membrane of epithelial cells. Analogous results have been obtained using a local potential fixation (patch clamp technique) in a cell-free system with ENaCs integrated into lipid bilayers and addition of actin microfilaments with subsequent exposure to cytochalasin D (Berdiev et al., 1996). Later, direct evidence was obtained that the C terminus of ENaC α-subunit can directly interact with actin (Mazzochi et al., 2006b). Finally, a set of recent studies has discovered a most important role of several small GTPases, influencing the actin cytoskeleton organization, in the control of ENaC activity (Pochynyuk et al., 2006; Saxena, Kaur, 2006; Karpushev et al., 2010, 2011).

The MDCK cell line initially deposited with the American Type Culture Collection (ATCC) is heterogeneous in its cell composition. In the 1980s, two sublines (MDCK1 and MDCK2) were obtained from this initial line by long-term serial passaging; these sublines considerably differ in their morphology, as well as their transport and electrophysiological characteristics (Barker, Simmons, 1981; Richardson et al., 1981). The most important electrophysiological parameter that characterizes both the barrier and transport properties of an epithelial layer is its transepithelial electric resistance (TEER), expressed in Ω/cm^2. The works referred to above have shown that the MDCK1 subline has a TEER of 3000–4000 Ω/cm^2 and MDCK2 of less than 100 Ω/cm^2. The initially deposited MDCK cell line (ATCC MDCK) also displays a low TEER, comparable to that of MDCK2 (Cereijido et al., 1978).

Thus, the MDCK1 cell monolayer is a cultured model of a "tight" transport epithelium and ATCC

MDCK, a cultured model of "permeable" transport epithelium. Comparative studies of these two cell sublines make it possible to reveal the specific features in their morphofunctional organization that show the drastic differences in the transporting characteristics of tight and permeable epithelia.

TEER characterizes the ion transport through an epithelial layer and is an integrated characteristic of a monolayer uniting the paracellular conductance of tight junctions and transcellular conductance of cell membranes. The transcellular component of the ion flux through MDCK monolayer is associated with the function of membrane transport proteins (first and foremost, ENaC and Na^+/K^+-ATPase), while the paracellular component is associated with the presence of particular claudin family members in the tight junctions of epithelial cells (Angelow, Yu, 2007; Krause et al., 2008). In particular, it is known that claudin-1 is involved in a barrier function in tight junctions (Inai et al., 1999; McCarthy et al., 2000) and claudin-2, on the contrary, is a pore-forming protein that mediates paracellular transport (Van Itallie et al., 2008). We selected these two claudins with opposite functions for immunofluorescence assay of their distributions in MDCK1 and ATCC MDCK cell monolayers.

The goal of this work was to study the functional relation between the AVP-induced dynamics of transepithelial resistance in the MDCK1 cell monolayer and the changes in spatial organization of their actin cytoskeleton. In addition, we examined the distribution of claudin-1 and -2 in the tight junctions of MDCK1 and ATCC MDCK cells by immunofluorescence assay in order to assess the potential contribution of paracellular component to the AVP-induced transepithelial transfer of ions and water through MDCK monolayer. The results suggest that the AVP-induced ion transport possesses a transcellular character and that the actin cytoskeleton plays a significant role in these transport processes.

MATERIALS AND METHODS

Cell cultures. Two MDCK cell subtypes were used in this work—the parental MDCK cell line, ATCC MDCK (cat. no. CCL-34), and MDCK1 line. The ATCC MDCK cells were obtained from the Russian Collection of Cell Cultures with the Institute of Cytology, Russian Academy of Sciences. MDCK1 cells were kindly provided by Prof. P. Deen (Nijmegen University, Netherlands). The cells were cultivated in the DMEM containing 50 μg/mL gentamicin and supplemented with 10% fetal bovine serum (Biolot, Russia) at 37°C in atmosphere of 5% CO_2. The culture medium was replaced every 2 days. In all experiments in this work, the MDCK cells were used as a confluent monolayer on days 3–4 of cultivation.

The cell medium was replaced with the DMEM free of fetal bovine serum 24 h before the experiment. The cell medium for the experimental groups was supplemented with AVP at a concentration of 10^{-6} M or forskolin at a concentration of 5×10^{-5} M. The incubation time was 15 min. The cells cultivated in an analogous manner but without adding physiologically active substances were used as a control.

Fluorescence microscopy. The distribution of F-actin, stained with the fluorescent dye rhodamine–phalloidin, in the MDCK1 cell line in the control and after exposure to AVP and forskolin was studied. The localization of the tight junction proteins, claudin-1 and -2, in the ATCC MDCK and MDCK1 cell lines was examined using an indirect immunofluorescent labeling.

For immunofluorescence assay, the cell monolayer grown on cover glasses was fixed with 2% paraformaldehyde solution in 0.01 M phosphate buffer (pH 7.4) for 10 min. The cells were then washed twice with 0.01 M phosphate buffer for 10 min and permeabilized with 0.1% Triton X-100 in 0.01 M phosphate buffer for 10 min. The nonspecific antibody binding was blocked by incubating cells in 2% bovine serum albumin solution in 0.01 M phosphate buffer for 10 min.

To detect actin structures, cells were incubated in rhodamine–phalloidin solution (1 : 200) in 0.01 M phosphate buffer for 30 min; washed with 0.01 M phosphate buffer; and embedded into DABCO medium, preventing discoloration.

Claudin-1 and -2, proteins characteristic of tight junctions, were detected by indirect immunolabeling with the antibodies to the corresponding antigens. The antibodies were diluted with bovine serum albumin solution in 0.01 M phosphate buffer. Polyclonal rabbit antibodies at a dilution of 1 : 200 were used for claudin-1 and -2. Cells were incubated with the primary antibodies for 1 h at room temperature, washed three times with phosphate buffer (10 min each), and incubated with secondary goat antibodies to the rabbit immunoglobulins conjugated with the fluorophore Alexa Fluor 488 at room temperature for 30 min. After triple washing with phosphate buffer, the cells on slides were embedded into DABCO.

Slides were examined using a Carl Zeiss Axioskop (Germany) fluorescence microscope with a 100× oil immersion objective lens.

In this work, we have quantitatively assessed the fluorescence intensity of filamentous actin in the control and experimental cells, separately analyzing the rhodamine–phalloidin fluorescence intensities in the apical and basal cell regions by refocusing the objective lens to the corresponding area of cell monolayer. For this purpose, 15–20 digital images were recorded for the control and experimental cells at the levels of their apical and basal cytoplasm using a Carl Zeiss Axioskop fluorescence microscope. All images were recorded under identical conditions without changing the digital camera and software settings. The images of control and experimental preparations were captured in a sin-

gle session. Then, the average numerical fluorescence intensity of the region occupied by cells for each image was computed using the ImageJ program; no graphical editing of images was used.

The statistical significance of the differences in the F-actin fluorescence intensity between experimental and control cells was confirmed by unpaired Student's *t*-test; the data were processed using the Statistica 8.0 program. Average fluorescence intensity for one image was regarded as an independent event.

Measuring transepithelial electric resistance (TEER). The TEER of the MDCK1 monolayer was measured to assess the characteristics of ion transport through this monolayer. For this purpose, the cells were cultivated on polyester membrane filters with a pore diameter of 0.4 μm.

TEER was measured using an EVOM2 (World Precision Instruments, Inc., United States) epithelial voltohmmeter equipped with a pair of Ag/AgCl electrodes. One electrode was immersed into the culture medium within a cylindrical fitting piece with a membrane filter and the other was placed in the medium outside the filter (in a well of a 24-well plate), keeping the same distance between the electrodes with an adapter. The immersion depth of electrode pair was controlled to be the same in all cases. When TEER was measured, a weak (12 μA) alternating current (12.5 Hz) was passed through the filter with a cell monolayer. This measurement scheme allows a diffusion flux of charged particles through the filter to be avoided. In addition, a noticeable influence of the own electric potential of the filter with cells in this case is absent, thereby giving a correct and reliable TEER value for the monolayer (Mishler et al., 1990). The electrodes were thoroughly washed in distilled water after each measurement.

The measured electric resistance between the internal and external wells reflects the total resistance of the porous membrane and the cell monolayer grown on it. Then, the resistance of an empty filter (150 Ω) was subtracted from the measured total value and the results converted into $\Omega\,cm^2$, thus obtaining the TEER estimate for epithelial monolayer.

Three independent experiments were performed for each variant and four membrane filters were used in each experiment.

Used materials and reagents: plasticware for cell cultivation (Orange Scientific, Belgium); polyester membrane filters (Corning Inc., United States); DMEM, fetal bovine serum, and phosphate buffer (Biolot, Russia); arginine vasopressin (ICN Pharmaceuticals, United States); forskolin and bovine serum albumin (Sigma, United States); paraformaldehyde (TAAB, Germany); primary polyclonal rabbit antibodies to claudin-1 and claudin-2 (Zymed Laboratories, United States); and rhodamine–phalloidin and secondary antibodies to rabbit immunoglobulins with Alexa 488 (Molecular Probes, United States).

RESULTS

Fluorescence microscopy of MDCK1 cell actin cytoskeleton. The actin cytoskeleton organization in the MDCK1 cells stained with rhodamine–phalloidin was examined. The filamentous actin was detectable in three main zones. The actin associated with microvilli localized in the apical region of the cytoplasm as well as the fine-fiber submembrane network of microfilaments. The actin connected with intercellular junctions was detectable in the central region of the cells near the cell boundaries. The basal region of MDCK1 cells contained stress fibers, ending with well-developed focal contacts. MDCK1 cells are also able to form flattened lamellipodia, associated with actin filaments. Lamellipodia are mainly present in single cells localized to the edge of the monolayer. Since the monolayer is rather thin (only 3–5 μm), the actins belonging to different pools (for example, stress fibers and the actin of intercellular junctions) are observable in the same images. Nonetheless, these three pools of actin filaments are readily distinguishable according to their structure (Fig. 1).

Study of the changes in the actin cytoskeleton organization of MDCK1 cells caused by the exposure to AVP and PKA activator, forskolin, as compared to the control conditions has shown that both agents decrease the actin fluorescence intensity. The apical actin network depolymerizes, the fluorescence in apical microvilli decreases, and a considerable number stress fibers in the basal cell region disappear. On the other hand, the actin fluorescence in the intercellular contacts almost does not change at all. Quantitative estimate of the average F-actin fluorescence intensity in the apical and basal regions of the control and experimental cells has shown that exposure to AVP (10^{-6} M, 15 min) decreases the fluorescence intensity in a statistically significant manner, namely, by 49.5 and 45.1% in the apical and basal regions, respectively, relative to the control (Fig. 2). The exposure to forskolin (5×10^{-5} M, 15 min) decreases the average fluorescence intensity by 45.6 and 52.7% in the apical and basal regions, respectively, as compared with the control (Fig. 3).

The changes in the TEER of the MDCK1 monolayer caused by AVP and forskolin. To assess the ion transport through the MDCK1 cell monolayer, TEER was measured under control conditions and after exposure to 10^{-6} M AVP or 5×10^{-5} M forskolin. Physiologically active substances in these experiments were added to the culture liquid from the basal side of cell monolayer.

In the control, the TEER of the MDCK1 cell monolayer exceeds 3000 $\Omega\,cm^2$; i.e., the MDCK1 line is functionally a tight epithelium (Tables 1 and 2).

AVP considerably (twofold) decreases the TEER of MDCK1 cell monolayer as early as the first 5 min of experiment (Table 1). A direct PKA activation by forskolin causes an even more pronounced decrease in TEER, amounting on the average to 67% (Table 2). A

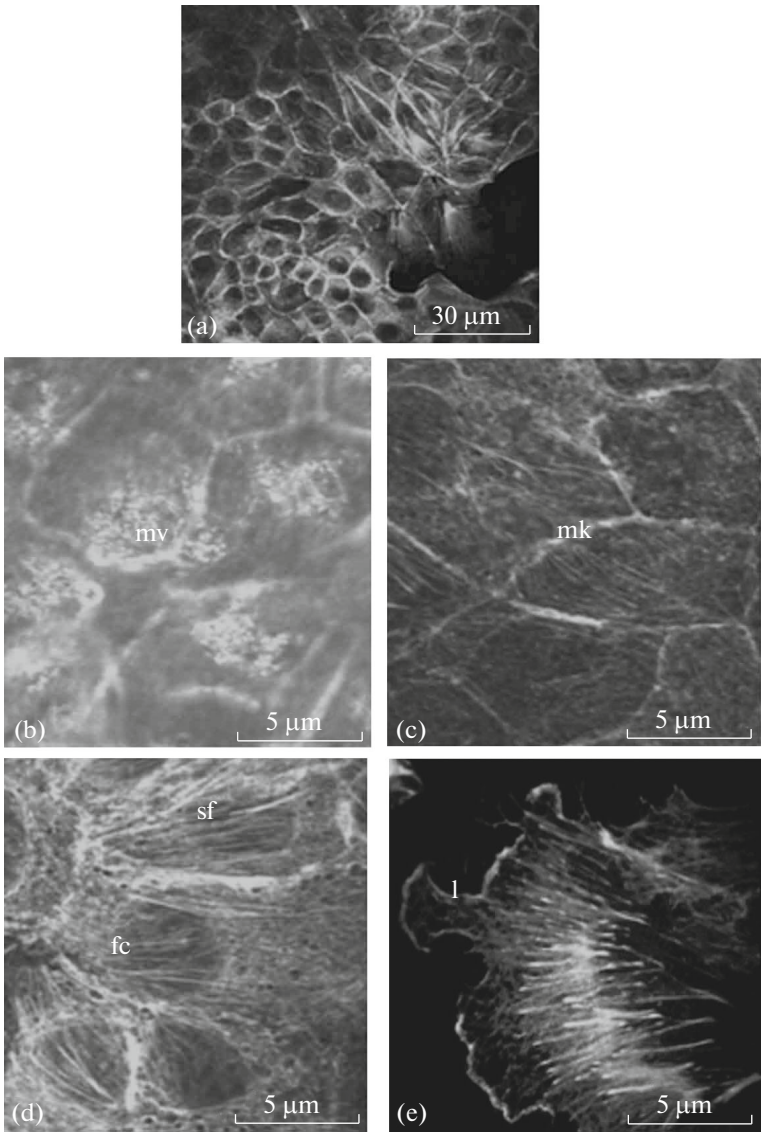

Fig. 1. Organization of the actin cytoskeleton in the MDCK1 cell monolayer under control conditions: (a) general view of the monolayer, (b) filamentous actin in bases of apical microvilli, (c) filamentous actin in intercellular junctions, (d) actin-containing stress fibers and focal contacts in basal cytoplasm, and (e) lamellopodium formed by a cell beyond the monolayer. Designations: mv, microvilli; ij, intercellular junctions; sf, stress fibers; fc, focal contacts; and l, lamellopodium. Staining with rhodamine–phalloidin.

reduced TEER level in both cases was retained after 10 and 15 min of experiment.

Immunofluorescent study of claudin-1 and -2 distributions in MDCK1 and ATCC MDCK. The paracellular permeability of an epithelial layer is mediated by claudins, localized to the tight junctions (Krause et al., 2008). Correspondingly, to study a potential contribution of the paracellular component to the observed changes in water and ion transport, we, first, studied the localization of claudin-1 and -2 in tight junctions of cells known to considerably differ in TEER, namely, ATCC MDCK (TEER of 30–70 $\Omega\,cm^2$) and MDCK1 (TEER over 3000 $\Omega\,cm^2$). Second, we studied the localization of two claudins in question after exposure to forskolin.

Claudin-1 was observed in tight junctions of both MDCK1 and ATCC MDCK cell monolayers. Pore-forming claudin-2 was detectable in ATCC MDCK cells and completely absent in MDCK1 cells (Fig. 4). Thus, the differences in TEER level between these two sublines are most likely explainable by the differences in claudin-2 localization in the tight cell junctions. However, immunofluorescence assay of these two claudins in MDCK1 cells exposed to forskolin at a concentration of 5×10^{-5} M did not detect any differences from the control cells: claudin-2 was also absent in the tight junctions of MDCK1 monolayer (Fig. 5). This suggests that the changes in TEER observed after the effect of forskolin are associated with the transcellular, rather than paracellular, monolayer permeability.

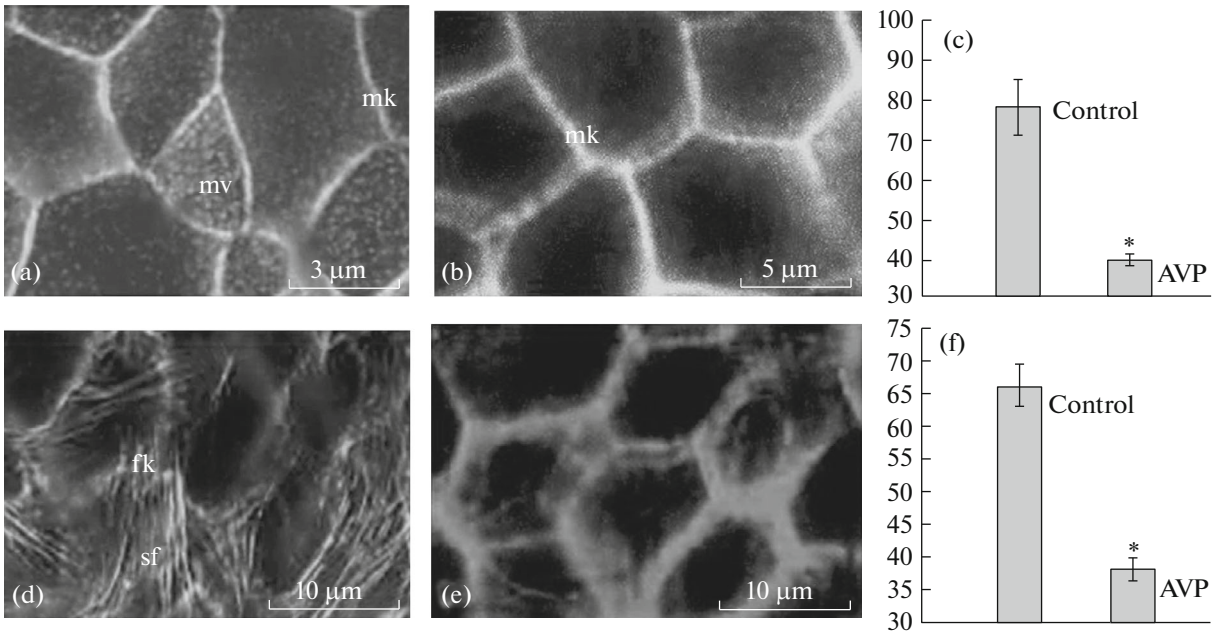

Fig. 2. Changes in actin cytoskeleton in (a–c) the apical and (d–f) basal regions of MDCK1 cell monolayer caused by exposure to AVP (10^{-6} M, 10 min): (a, d) control, (b, e) AVP, and (c, f) the actin fluorescence intensity in the control and after exposure to AVP. Vertical axis shows the actin fluorescence intensity in MDCK1 cells (arbitrary units), vertical intervals, standard error of the mean, and asterisk, statistically significant difference at $p < 0.01$; $n = 15$ in both cases. For designations, see Fig. 1.

DISCUSSION

Transepithelial electric resistance and its dynamics when epithelial cells are exposed to various biologically active substances are a very important functional characteristic for the barrier and transport properties of an epithelial layer. Our study has confirmed that the TEER of the MDCK1 cell monolayer is 3000–4000 $\Omega\,cm^2$ and is a functionally "tight" epithelium. AVP causes a rapid drop in the TEER of MDCK1 cells to 1500–1800 $\Omega\,cm^2$; that is, a twofold increase in the ion conductance of epithelium is observed. An even more considerable decrease in TEER was caused by forskolin, an activator of the PKA signaling pathway. Several earlier studies have demonstrated that AVP stimulates transepithelial sodium ion flux in AVP-sensitive epithelia both in vivo and in vitro (Frindt, Burg, 1972; Garty, Edelman, 1983; Jeffries et al., 1988; Stockand, 2010).

Table 1. TEER dynamics in MDCK1 cell monolayer in control and after exposure to AVP

Duration of experiment, min	Transepithelial electric resistance, $\Omega\,cm^2$		
	control	arginine vasopressin, 10^{-6} M	
	filter 1	filter 2	filter 3
0	3833	4200	4367
5	4100	1867	1967
10	4033	1933	2067
15	3233	1900	1967

Table shows a representative result of one of the four replicate experiments.

The general physiological literature regards mainly TEER as a criterion for the integrity and confluence of a cell monolayer (for example, when assessing the cytotoxic effect of various substances), as well as an indicator of the barrier properties of tight intercellular junctions (Benson et al., 2013; Srinivasan et al., 2015). However, the transepithelial resistance is an integral characteristic that combines the paracellular conductance of tight junctions and transcellular conductance of cell membranes. Actually, TEER at each time moment is simultaneously determined by the state of cell systems providing the ion transport and functional "density" of tight junctions. Strictly speaking, this is why the drastic change in TEER observed in this work is insufficient for a solid conclusion to be drawn on the routes of an AVP-induced ion flux through a cell monolayer.

According to the current paradigm, the paracellular permeability of epithelia is determined by localization of various claudins in their tight junctions (Anderson et al., 2004; Elkouby-Naor, Ben-Yosef, 2010). In particular, there is convincing evidence that claudin-1, a member of this protein family, is a structural protein of tight junctions, whereas claudin-2, another member of this family, is a pore-forming protein determining the paracellular permeability of an epithelial layer (Krause et al., 2008; van Itallie et al., 2008).

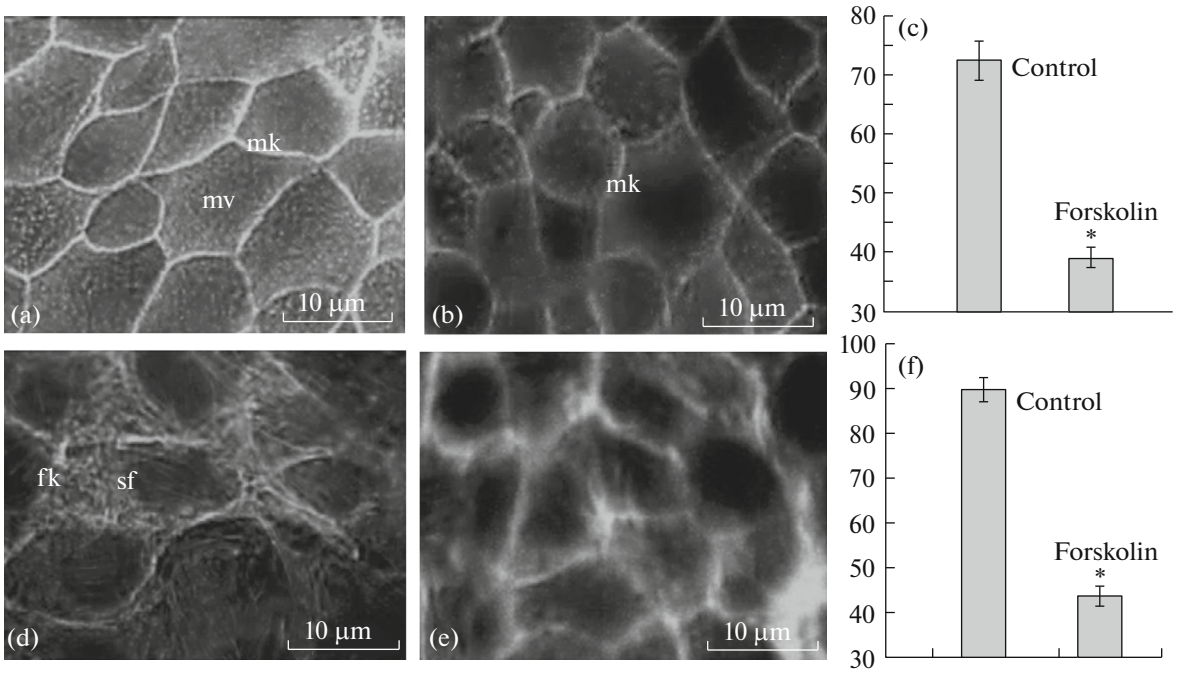

Fig. 3. Changes in actin cytoskeleton in (a–c) the apical and (d–f) basal regions of MDCK1 cell monolayer caused by exposure to forskolin (5×10^{-5} M, 10 min): (a, d) control, (b, e) forskolin, and (c, f) the actin fluorescence intensity in the control and after exposure to forskolin. Vertical axis shows the actin fluorescence intensity in MDCK1 cells (arbitrary units), vertical intervals, standard error of the mean, and asterisk, statistically significant difference at $p < 0.01$; $n = 15$ in both cases. For designations, see Fig. 1.

Based on this literature data, we have assumed that, if AVP-induced stimulation of the ion transport in the studied cells is to a considerable degree mediated by a change in the paracellular route, this should be reflected by a change in the level of expression of pore-forming claudin-2 in tight junctions. In this connection, we used immunofluorescence assay to determine the distributions of claudin-1 and -2 in the intact MDCK1 cells (a high TEER) and ATCC MDCK (a low TEER) and after exposure to forskolin. It has been shown that claudin-1, as a structural protein determining the architecture of tight junctions, is present in all cell monolayers under all experimental conditions. On the contrary, claudin-2 is abundant in the tight junctions of ATCC MDCK cells but is absent in MDCK1 cells. This observation agrees with the earlier published data (Furuse et al., 2001) and confirms that there is a functional linkage between the TEER level in an epithelial layer and the claudin-2 localization there. However, no changes in the distribution of these claudins were observable when MDCK1 cell monolayer was exposed to forskolin. This result is clear evidence that the AVP-induced sodium ion flux in a MDCK cell monolayer is directed transcellularly, rather than paracellularly.

We have also studied the actin cytoskeleton organization in the intact MDCK1 cells and the cells exposed to AVP and forskolin by fluorescence microscopy and observed that both AVP and forskolin cause a statistically significant F-actin depolymerization in all cytoplasm domains except for the intercellular junctions. This is another fact suggesting that tight junctions (unlike the membrane and cytoplasmic structures) are not active players in the AVP-induced ion transport in MDCK1 cells.

The cell actin cytoskeleton is a highly dynamic system of microfilaments intensively regulated by cell signaling pathways. As a rule, actin cytoskeleton remodeling is accompanied by a change in the cell functional state, contributing to both the cell structural remodeling and changes in the cascades of protein–protein interactions (Pinaev, 2009). In particular, actin depo-

Table 2. TEER dynamics in MDCK1 cell monolayer in the control and after exposure to forskolin

Duration of experiment, min	Transepithelial electric resistance, Ω cm^2		
	control	forskolin, 5×10^{-5} M	
	filter 1	filter 2	filter 3
0	4200	4433	4400
5	4333	1600	1500
10	4366	1466	1400
15	4366	1433	1366

Table shows a representative result of one of the four replicate experiments.

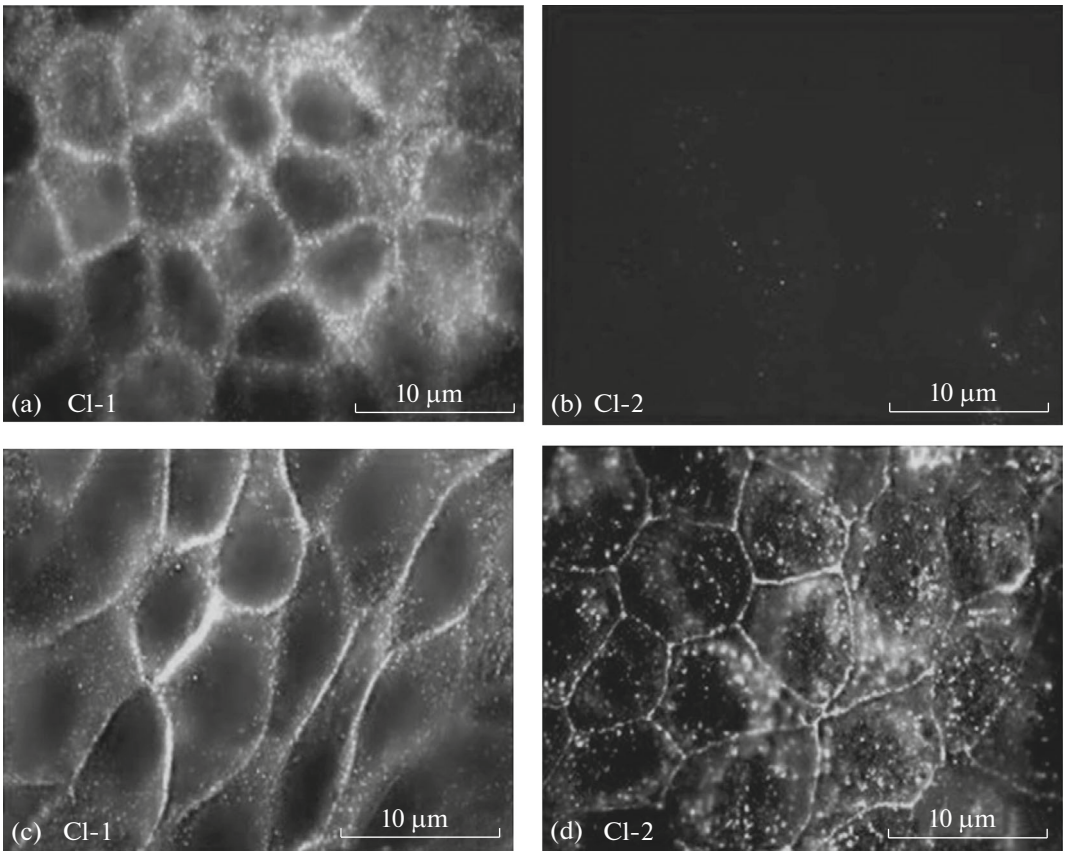

Fig. 4. Localizations of claudin-1 and claudin-2 in (a, b) MDCK1 and (c, d) ATCC MDCK cells. Pore-forming claudin-2 is absent in MDCK1 cells, while both proteins are present in ATCC MDCK cells. Designations: Cl-1, claudin-1; Cl-2, claudin-2.

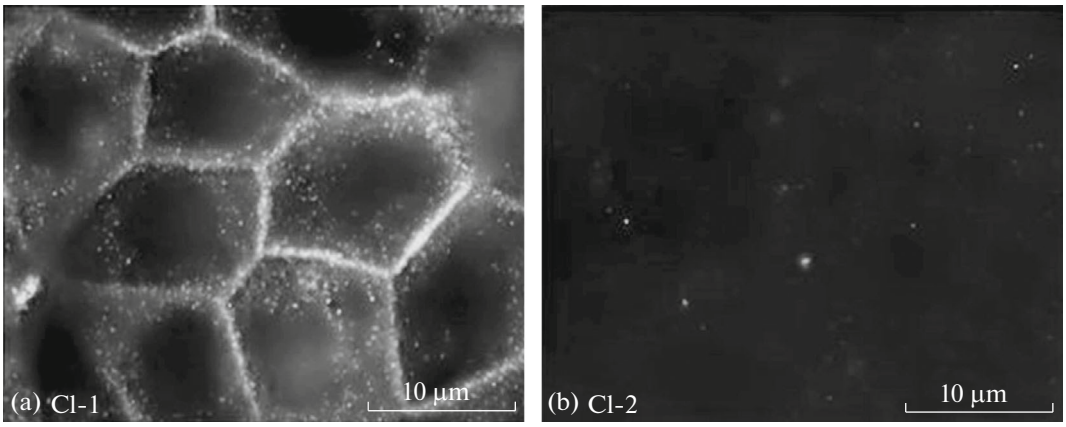

Fig. 5. Localizations of (a) claudin-1 and (b) claudin-2 in tight junctions of MDCK1 cells after exposure to forskolin (5×10^{-5} M, 10 min). No differences are observed in the distribution of these claudins as compared with the control (see Figs. 4a and 4b). Designations: Cl-1, claudin-1; Cl-2, claudin-2.

lymerization in response to AVP has been observed in several vasopressin-sensitive epithelia (Hays et al., 1993; Simon et al., 1993; Gorshkov, Komissarchik, 1997; Klussmann et al., 2001).

Questions of the particular functional role and the molecular and signaling mechanisms determining involvement of actin cytoskeleton in the AVP-induced stimulation of ion and water transepithelial transport have been actively discussed for several decades. In general, it is well known that the AVP-induced transcellular sodium flux is mediated by ENaCs in the apical epithelial cell membrane and Na^+/K^+-ATPase in

their basolateral membrane. According to the available data, the potential "throughput" of Na^+/K^+-ATPase for sodium is several times higher than for ENaC; therefore, the sodium input to cells through ENaCs is the limiting component in the transepithelial sodium flux (Niisato, Marunaka, 1999; Ashkroft, 2000). Correspondingly, ENaCs are the primary target for the cell regulatory systems that control the sodium permeability of an epithelium (Loffing, Korbmacher, 2009).

Two main hypotheses explain the role of actin in the ENaC function (which, in fact, are not mutually exclusive): (1) the possible effect of microfilaments on the open (closed) state of the channel, i.e., directly on the conformation of ENaC subunits and (2) the possible involvement of actin cytoskeleton in maintaining the balance between the ENaC integration into the plasma membrane and ENaC internalization, i.e., in the processes controlling the number of channels in the membrane.

The results of several studies in cell-free systems (artificial lipid bilayers with incorporated ENaCs) favor the first hypothesis. Addition of actin monomers to this system and its rapid assembly into short microfilaments significantly changes the ENaC electrophysiological (patch clamp) characteristics, namely, considerably increases the average time of a channel open state (Berdiev et al., 1996; Jovov et al., 1999). In these works, they also modeled the effects of several cellular factors that influence the actin functional state. In particular, it has been shown that the addition of ARP, PKA, or an actin-binding protein, gelsolin, to the lipid bilayer–ENaC–actin system causes further ENaC activation (Berdiev et al., 1996). However, calcium blocks actin-dependent ENaC activation in the considered cell-free system (Berdiev et al., 2001). As was later shown, the C terminus of ENaC α-subunit (amino acid residues 631–644), which is able to directly interact with actin, is responsible for an actin-dependent modulation of the ENaC properties (Copeland et al., 2001; Mazzochi et al., 2006b).

Despite the importance and reliability of the above data obtained in cell-free systems, it is evident that they are unable to fully simulate the role of actin cytoskeleton in transepithelial transport and require verification and clarification using cell models, since cells are a considerably more complex and multifactorial system as compared with the lipid bilayers in question. There is clear evidence that stimulation of the PKA signaling pathway, which mediates the effect of AVP, causes an increase in the number of sodium channels in the apical membrane of epithelial cells. This evidence was first obtained using the patch clamp technique (Marunaka, Eaton, 1991). Note that the authors of this work traced the appearance of local membrane hot spots with high sodium permeability caused by the effect of AVP on collecting duct cells. This observation is precisely comparable to the appearance of the areas displaying high water permeability containing water channels in the apical membrane of AVP-sensitive epithelia. Such regions have been earlier detected with the help of freeze-fracture electron microscopy (Muller, Kachadorian, 1984; Komissarchik, Snigirevskaya, 1991). This suggests that the AVP-driven regulation of water and sodium transepithelial transport follows similar cellular mechanisms, albeit targeted to different proteins, aquaporin-2 (for water) and ENaC (for sodium).

Later studies confirmed that there is an AVP-induced increase in the number of ENaC molecules in the epithelial cell apical membrane in vitro for biotinylated ENaC α-, β-, and γ-subunits by Western blotting (Butterworth et al., 2005).

The role of actin cytoskeleton in the ENaC function has been repeatedly studied using an agent depolymerizing microfilaments, cytochalasin D. Several studies have shown that cytochalasin D may have an opposite effect on the activity of ENaCs depending on the time of its action. A short-term (5 min) exposure to cytochalasin D activates the transepithelial sodium flux and increases the average time when ENaCs are opened (Cantiello et al., 1991). On the contrary, a longer cell incubation with cytochalasin D (1 h and more) inhibits ENaC activity and completely levels the stimulatory effect of PKA or the effect of hyperosmotic medium on the basolateral surface on the sodium flux through the cell monolayer (Prat et al., 1993; Rehn et al., 1998). An analogous result (ENaC inhibition) has been observed after 1-h exposure to cytochalasin E (Reifenberger et al., 2014).

The authors of these papers explain their observations by arguing that implementation of the ENaC function (open state of the channel) and, possibly, successful ENaC integration into the apical cell membrane require its interaction with short actin filaments, which functionally differ from highly polymerized F-actin. Such filaments result from an incomplete decomposition caused by a short-term action of cytochalasin D. ENaC inactivation in the lipid bilayer–ENaC–actin system caused by addition of the actin-cutting protein gelsolin is explainable in a similar manner (Berdiev et al., 1996).

Note that our results on the considerable AVP-induced actin depolymerization in MDCK cells match well the above-described data on the effect of a short-term (5 min) exposure to cytochalasin D on the ENaC activity.

The inhibition of ENaCs by the actin-binding proteins filamin (Wang et al., 2013) and cortactin in complex with Arp2/3 (Ilatovskaya et al., 2011) has been reported. These results are explainable by both direct ENaC interactions with filamin and cortactin demonstrated in the afore-mentioned studies and an actin-organizing role of these proteins. It is well known that filamin enhances cross linking and stabilization of actin networks (Nakamura et al., 2011) and cortactin, Arp2/3-dependent actin polymerization (Ammer,

Weed, 2008). Thus, these proteins prevent the submembrane F-actin from remodeling into short filaments, interaction with which is most likely necessary for a normal ENaC function.

A long-term incubation of the ENaC-expressing cells with cytochalasins most likely leads to a complete F-actin depolymerization, making impossible the ENaC channel function and/or ENaC integration into the membrane, thereby inhibiting the transepithelial sodium transport (Prat et al., 1993; Rehn et al., 1998; Reifenberger et al., 2014).

Currently, it is well known that the spatial organization of actin cytoskeleton in the cell is controlled by several small GTPases, first and foremost, RhoA, Rac1, and Cdc-42 (Hall, Nobes, 2000). Several recent studies have demonstrated that these small GTPases (RhoA and Rac1) as well as the GTPases Rab11 and K-Ras in an active GTP-associated form increase the sodium transport activity through ENaCs (Staruschenko et al., 2004; Saxena, Kaur, 2006; Pochynyuk et al., 2006; Karpushev et al., 2011). According to these data, K-Ras, an active small GTPase, increases the probability of the channel to be open without altering the number of channels in the cell membrane (Staruschenko et al., 2004, 2005), whereas the GTPases Rab11, RhoA, and Rac1 influence the ENaC integration into the apical membrane (Saxena, Kaur, 2006; Pochynyuk et al., 2006; Karpushev et al., 2011). This is additional evidence of a significant role of actin cytoskeleton in transepithelial sodium transport that is uniquely intricate and involves multiple regulation pathways.

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