Protamine-Induced Epithelial Barrier Disruption Involves Rearrangement of Cytoskeleton and Decreased Tight Junction-Associated Protein Expression in Cultured MDCK Strains

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ABSTRACT. Natural and synthetic polycationic proteins, such as protamine, have been used to reproduce the tissue injury and changes in epithelial permeability caused by positively charged substances released by polymorphonuclear cells during inflammation. Protamine has diverse and often conflicting effects on epithelial permeability. The effects of this polycation on the distribution and expression of tight junction (TJ)-associated proteins have not yet been investigated. In this work, we examined the influence of protamine on paracellular barrier function and TJ structure using two strains of the epithelial Madin-Darby canine kidney (MDCK) cell line that differed in their TJ properties (“tight” TJ-strain I and “leaky” TJ-strain II). Protamine induced concentration-, time- and strain-dependent alterations in transepithelial electrical resistance (R) only when applied to apical or apical+basolateral monolayer surfaces, indicating a polarity of action. In MDCK II cells, protamine (50 μg/ml) caused a significant increase in R, that returned to control values after 2 h. However, the treatment of this MDCK strain with a higher concentration of protamine (250 μg/ml) significantly decreased the R after 30 min. In contrast, treated MDCK I monolayers showed a significant decrease in R, after apical treatment with protamine at both concentrations. The protamine-induced decrease in R, was paralleled by an increase in the phenol red basal-to-apical flux in both MDCK strains, suggesting disruption of the paracellular barrier. Marked changes in cytoskeletal F-actin distribution/polymerization and a significant reduction in the junctional expression of the tight junctional proteins occludin and claudin-1 but subtle alterations in ZO-1 were observed following protamine-elicited paracellular barrier disruption. In conclusion, protamine induces alterations in the epithelial barrier function of MDCK monolayers that may involve the cytoskeleton and TJ-associated proteins. The various actions of protamine on epithelial function may reflect different degrees of interaction of protamine with the plasma membrane and different intracellular processes triggered by this polycation.

Key words: epithelial barrier/protamine/MDCK strains/tight junction/cytoskeleton

Introduction

One of the most important attributes of epithelia is their ability to function as a barrier that prevents the permeation of noxious agents to the internal milieu and, at the same time, contributes to the vectorial transepithelial transport of nutrients, electrolytes and water (Madara, 1998). These roles depend on membrane specializations in the cell-cell contact region which forms the junctional complex, i.e. the tight junction (TJ), the zona adherens and desmosomes. The TJ has been considered the specific anatomical site corresponding to the route-limiting permeability barrier of epithelia since cellular membranes of adjacent cells are at their closest apposition in this region (Anderson, 2001; Schneeberger and Lynch, 2004).

A number of experimental interventions and pathophysiological states can alter the paracellular permeability and modify the expression, cellular distribution and/or phosphorylation of several TJ-associated proteins, and also change the functional interactions between these proteins and the cytoskeleton (Madara et al., 1986; Polak-Charcon, 1992; Collares-Buzato et al., 1994, 1998a, 1998b; Jepson et al., 1995; Collares-Buzato, 2001; Tsukita and Furuse, 2002;...
The effect of natural and synthetic polycationic proteins, such as protamine and poly-L-lysine, upon epithelial permeability has been investigated in several tissues in vivo and in vitro. These polycations have been used to reproduce the charge-mediated effects, such as damage to the cell membrane and increased capillary permeability, of some positively-charged substances released by polymorphonuclear cells during inflammation (Fromm et al., 1985; Bentzel et al., 1987; Chang et al., 1987; Peterson and Gruenhaupt, 1990; McEwan et al., 1993). In the case of protamine, understanding its mechanisms of action is of interest because of its clinical use to reverse the anticoagulant effect of heparin, and also its possible involvement in the development of nephrotoxicity and noncardiogenic pulmonary edema (Olinger et al., 1980; Fromm et al., 1985).

Protamine has diverse, sometimes conflicting, effects on epithelial permeability since it may increase or decrease the transepithelial electrical resistance, depending on the tissue type, and this may or may not be accompanied by changes in apical membrane resistance. These results are difficult to compare not only because of differences in the animal species and cell types used, but also because of the rather wide range of concentrations protamine tested. Alterations in the structure and function of TJ have been indicated as a possible cause of the changes in epithelial permeability. However, the effect of protamine upon the distribution and expression of TJ-associated proteins has not yet been investigated.

In this work, we examined the action of protamine on paracellular barrier function and on the structure and function of the TJ using cultured Madin-Darby canine kidney (MDCK) cells as an in vitro model system of epithelia. Two strains of MDCK cells were used: strain I, which displays characteristics consistent with those of “tight” epithelia (Rt ~ 5000 Ω.cm²) and strain II, which has “leaky”, cation-selective tight junctions with Rt values of ~ 200 Ω.cm² (Collares-Buzato et al., 1994, 1998a, 1998b).

Materials and Methods

Reagents

Sterile plastic material for cell culture was supplied by Nunc (Roskilde, Denmark) or Corning (Corning, NY). Cell culture media and supplements were purchased from Cultilab or Nutricell (Campinas, SP, Brazil). Primary and secondary antibodies were supplied by Zymed (San Francisco, CA) or Sigma (St. Louis, MO). All other chemicals and reagents were supplied by Sigma and Merck.

Cell culture

Two strains of MDCK renal epithelial cells were studied in this work: the “low resistant” MDCK II was obtained from the Adolfo Lutz Institute (São Paulo, SP, Brazil) and the “high resistant” MDCK I was kindly donated by Dr. Barry H. Hirst (University of Newcastle upon Tyne, UK). The cells were grown in plastic flasks at 37°C, in an humidified atmosphere of 5% CO2/air, with Eagle’s minimum essential medium (EMEM) supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. The culture medium was changed every 48 h and, at confluence, the cells were passaged weekly with trypsin (2.5 mg/ml) and EDTA (100 μg/ml). For the experiments, cells were seeded at a high density (0.5×10⁶ cells/cm²) on to specific tissue inserts incorporating a permeable inorganic membrane filter (Anocell, Nunc, membrane area of 0.78 cm², or Falcon, P.E.T., Becton Dickinson, Franklin, Lakes, NJ, membrane area 0.33 cm²) and grown in 6-well or 12-well plates with supplemented EMEM for 4 days.

Treatment with protamine

Protamine base (Sigma) was diluted in buffered Krebs-Ringer (27.4 mM NaCl, 1 mM KCl, 0.2 mM MgSO4·7H2O, 0.06 mM KH2PO4, 0.06 mM Na2HPO4·H2O, 2.8 mM Trizma base, 0.56 mM CaCl2·2H2O, and 1.8 mM glucose) to a final concentration of 50 μg/ml or 250 μg/ml (pH 7.4, adjusted with HCl after protamine addition). The culture media bathing the monolayers were gently withdrawn and replaced by the buffered Krebs-Ringer containing or not protamine. The protamine-containing Krebs-Ringer was applied to the apical and/or basolateral surfaces of the MDCK II monolayers. MDCK I monolayers were treated with protamine only on their apical surface. The control cells received the same volume of Krebs-Ringer without the polycation. The cells were maintained at 37°C throughout the experiment.

Measurement of the transepithelial electrical resistance (Rt)

Transepithelial electrical resistance (Rt) was measured across MDCK monolayers using two Ag/AgCl “chopstick” electrodes coupled to a combined voltmeter and constant current source (EVOM, World Precision Instruments, UK). Rt measurements were obtained at 10, 30, 60 and 120 min after treatment.

Immunostaining of tight junction-associated proteins

Following exposure to protamine, some monolayers were prepared for analysis of the expression and distribution of the tight junction-associated proteins occludin, ZO-1 and claudin-1. MDCK cells were fixed and immunostained for these proteins using a standard indirect immunofluorescence protocol (Collares-Buzato et al., 1998a, 1998b). Briefly, monolayers were fixed and permeabilized with methanol at −20°C and then incubated overnight at 4°C with primary antibodies against occludin (dilution 1:100, polyclonal rabbit anti-occludin antibody, Zymed, USA), ZO-1 (dilution 1:50, polyclonal rabbit anti-ZO-1 antibody, Zymed, USA) or claudin-1 (dilution 1:50, polyclonal rabbit anti-claudin-1 antibody, Zymed, USA), followed by a 2 h incubation with FITC-conjugated specific
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secondary antibody (Sigma) at room temperature (RT), in the dark. Subsequently, monolayers were washed several times with phosphate-buffered saline solution (10 mM PBS, pH 7.4, 136 mM NaCl, 2.6 mM KCl, 8.1 mM Na$_2$HPO$_4$, 1.4 mM KH$_2$PO$_4$), and then detached from the Anocell inserts and mounted with Vectashield (Vector Laboratories, Inc., Burlingame, CA). Cell staining was detected by confocal laser scanning microscopy (CLSM, Bio-Rad MRC 1024, Bio-Rad, Richmond, CA). To allow comparison between the treated and control groups, the microscopic examination of both groups was done in the same experimental session. Staining was absent from negative control inserts in which the primary antibodies were omitted. The degree of emitted fluorescence from the pancreas sections of the control and treated groups was measured using a software provided by the CLSM and expressed as arbitrary fluorescence units.

**Cytochemistry for F-actin**

Following treatment with protamine, MDCK cells were fixed with 3.7% formalin solution (in 10 mM PBS, pH 7.4) for 30 min, permeabilized with 0.1% Triton X-100 for 10 min (in 10 mM PBS, pH 7.4) and incubated for 2 h with TRITC-labelled phalloidin (dilution 1:50, in 10 mM PBS, pH 7.4) at 37°C, in the dark. The monolayers were then washed several times with PBS buffer (pH 7.4), detached from the Anocell inserts, and mounted with Vectashield. Cell staining was detected by CLSM. Double labeling of F-actin and occludin was also done in some methanol-fixed MDCK II monolayers. After immunolabelling for occludin as described above, the monolayers were then 2 h incubated with TRITC-phalloidin before mounting on coverslip. To allow comparison between the treated and control groups, the microscopic examination was done in the same experimental session.

**Phenol red flux**

The phenol red flux was assayed as described before, with some modifications (Jovov et al., 1991). After 4 days in culture, MDCK cells seeded on permeable tissue culture supports were transferred to a new plate containing buffered Krebs-Ringer and phenol red (20 mg/ml) (basolateral surface). The apical medium was replaced by a Krebs-Ringer solution without phenol red. The apical surface of the treated group received Krebs buffer containing protamine, while the control group received the same volume of Krebs without polycation. After the incubation period, 0.4 ml samples were taken from the apical surface and, at the end of the 2 h incubation, the whole basal bathing solution was collected. All of the samples were read at 492 nm in a microplate reader (Labsystems). The basal-to-apical phenol red flux (Fb→a) was expressed as percentage of the absorbance values of the basal sample (taken as 100% dye concentration). As positive control for changes in paracellular permeability, some MDCK II monolayers were treated with Krebs-Ringer (without Ca$^{2+}$) containing 1 mM EDTA.

**Analysis of cell viability**

The potential cytotoxic effect of protamine in MDCK cells was analyzed using the neutral red dye assay (Borenfreund and Puerner, 1984). For this procedure, MDCK cells were seeded in 96-well plates at a density of 1.96×10$^4$ cells per well. After 4 days in culture, the EMEM was replaced by buffered Krebs-Ringer containing different concentrations of protamine in the treated group, and by Krebs-Ringer without protamine in the control group. Following a 2 h treatment at 37°C, the buffer was removed and 200 μl of Krebs containing neutral red (5 μg/ml) were added to each well. The plate was incubated for 3 h at 37°C after which the buffer containing the dye was removed and the wells were washed twice with formol-calcium (40% formaldehyde, 1% anhydrous calcium chloride, w/w). Finally, 200 μl of an acetic acid-saline solution (1 ml of glacial acetic acid in 100 ml of ethanol) were added to each well followed by incubation for 15 min at RT. The plate was read at 540 nm in a microplate reader (Labsystems). As a positive control for cytotoxicity, some monolayers were treated with 0.5% Triton X-100 (diluted in Krebs-Ringer, pH 7.4) for 2 h at 37°C. The cell viability was expressed as a percentage of the mean absorbance values obtained for the control wells (taken as 100%).

**Statistical analysis**

All numerical results were expressed as the mean±standard error (SE). Statistical comparisons between two groups were done using Student’s t-test. For multiple comparisons, the significance was assessed by ANOVA followed by the Bonferroni test. The level of significance was set at P≤0.05.

**Results**

Fig. 1 shows the time-course and dose-dependence for the apical treatment with protamine in MDCK II and I monolayers. In MDCK II cells, protamine (50 μg/ml) increased the transepithelial resistance (Rt) by approximately 50% after 10 min of exposure when compared to the control (Fig. 1a). However, treatment with a higher concentration of protamine (250 μg/ml) significantly decreased the Rt by approximately 82% compared to the control, after a 30 min exposure (Fig. 1b). In contrast to MDCK II cells, protamine-treated MDCK I monolayers showed a significant decrease in Rt after exposure to protamine at both concentrations: the lower concentration (50 μg/ml) decreased the Rt by approximately 74% after a 30 min of exposure (Fig. 1c), while the higher concentrations (250 μg/ml) reduced the Rt by approximately 82% after 10 min when compared to the control cells (Fig. 1d).

Protamine caused the significant alterations in Rt of MDCK II monolayers only when applied to the apical surface (Fig. 2). The exposure to protamine (50 μg/ml) at the apical or apical+basal surfaces increased significantly the Rt by approximately 43% or 28%, respectively, in comparison
with control and basal-treated cells (P<0.05). Meanwhile, higher concentration of protamine (250 µg/ml) at apical or apical+basal surfaces decreased significantly the Rₜ by approximately 88% or 82%, respectively, in comparison with control and basal-treated cells (P<0.001) (Fig. 2). So, the effect of protamine in these cells is polarized, dependent on the addition of this polycation to the cell apical surface.

The phenol red flux assay was used to assess whether protamine specifically affected the paracellular barrier function in MDCK II and I monolayers. MDCK II cells exposed to protamine (50 µg/ml) for 1 h or 2 h showed no significant change in the phenol red Fₘ,ₙ, when compared to the controls (Control 1 h=3.50±0.58 (5)% vs Treated PRO50, 1 h=2.84±0.52 (4)%; Control 2 h=3.12±0.67 (5)% vs Treated PRO50 2 h=3.36±0.30 (4)%). MDCK II treatment with a higher concentration (250 µg/ml) of protamine for 2 h resulted in an approximately 3.5-fold increase in the phenol red Fₘ,ₙ, similarly to that observed with EDTA treatment (Control=3.12±0.67 (5)% vs Treated PRO250=19.69±3.11 (3)% (Control vs Treated PRO250, P<0.001; Control vs Treated EDTA, P<0.001). In accordance with the literature (Peterson and Gruenhaupt, 2003), these results suggest that the effect of protamine is concentration- and time-dependent. The MDCK II columns are the means±SE of 6–12 monolayers per group from 2–4 independent experiments. The MDCK I columns are expressed as the means±SE of five monolayers per group from two independent experiments. *P<0.001 and **P<0.01 (ANOVA and Bonferroni’s test).
compared to the control group. These significant alterations in Rt (Control=3.15±0.27 (4)% increased the basal-to-apical phenol red flux in this strain (18); protamine (50 μg/ml tested (250 μg/ml) had any cytotoxic effect on cultured cells (control absorbance=0.065±0.002 (18); protamine (250 μg/ml)=0.09±0.009 (18); Triton X-100 0.5% (positive control)=0.0005±0.0004 (9)). So, changes observed in epithelial permeability induced by protamine were not due to cell death.

To assess whether protamine altered the cytoskeleton in the two MDCK strains, monolayers were stained with FITC-conjugated phalloidin to reveal F-actin (Figs. 3 and 4). Following a 2 h exposure to 50 μg of protamine/ml, MDCK II cells showed a marked disorganization of the cytoskeletal microfilaments seen as F-actin condensation in some areas of intercellular contact (Fig. 3). This cytoskeletal alteration was only observed when protamine was applied to the apical and apical+basolateral surfaces (Fig. 3c and 3d). After a 1 h exposure to this same concentration of protamine, the cells already showed disorganization of the F-actin microfilaments, but a lower number of cells were affected (Fig. 3, insert). Fig. 4 shows that treatment with protamine at a concentration of 250 μg/ml caused a greater alteration in the cytoskeleton compared to that seen with 50 μg/ml. At this higher concentration, MDCK II cells showed an intense staining for F-actin at the apical surface and in some areas of the plasma membrane at the cell-cell contact region (Fig. 4a, b). In addition, the most affected cells displayed a more rounded shape. In MDCK I cells, protamine (250 μg/ml) produced similar effects to those seen in MDCK II cells, although a lower number of cells was affected and at lesser extension in comparison with the former strain (Fig. 4c, d).

The expression and localization of the TJ-associated proteins occludin, claudin-1 and ZO-1 in MDCK II and MDCK I strains after treatment with protamine were analyzed by immunofluorescence (Figs. 5–9). As shown in Fig. 5, a 2 h exposure to 50 μg of protamine/ml did not significantly alter the immunostaining for these junctional proteins in MDCK II cells. Nevertheless, 250 μg of protamine/ml for 30 min induced a significant decrease in the junctional content of occludin in both MDCK strains (Fig. 6). Some cells of the monolayers were more affected than others and showed a complete lack of occludin in the junctional area (Fig. 6). Double-staining for occludin and F-actin microfilaments showed that some treated MDCK II cells had marked condensation of the cytoskeletal microfilaments in regions of intercellular contact associated with either a complete lack of occludin or an intense punctual reaction for this protein in the junctional area (Fig. 7).

As indicated in Fig. 8, immunostaining for claudin-1 showed that protamine (250 μg/ml)-treated MDCK II cells had a marked decrease in the junctional reaction for this protein. These protamine-treated strain II cells also had a greater amount of punctuate labelling within the cytoplasm (sometimes resembling vesicles) in comparison with the control cells (Fig. 8). These data may suggest the internalization of claudin-1 after the exposure to protamine.

Protamine produced no significant alterations in the junctional expression and distribution of ZO-1 in either strain of MDCK cells (Figs. 5 and 9), although in MDCK II cells...
treated with 250 µg protamine/ml, the ZO-1 immunoreaction was less evenly distributed at cell periphery in comparison with the control cells. Overall, MDCK I monolayers were generally less affected in comparison with MDCK II cells since MDCK I showed only marked alterations in immunolabelling for occludin but not for claudin-1 or ZO-1 after treatment with protamine (250 µg/ml) (Figs. 6, 8 and 9).

**Discussion**

Polycations are well known to affect tissue function. A number of cationic proteins released from polymorphonuclear leukocytes (PMN) such as the major basic protein (MBP), the eosinophil cationic protein (ECP) and the heparin-binding protein (HBP) may contribute to the pathogenesis of the inflammatory state by inducing tissue injury, an increase in vascular permeability, and edema at sites of inflammation (Henson and Johnston, 1987; Gautam et al., 2001; Jones et al., 2001). There is in vivo and in vitro evidence showing that those effects involve disruption of the epithelial barrier function and cytoskeletal rearrangement (Frigas et al., 1980; Motojima et al., 1989; Coyle et al., 1993; Gautam et al., 2001). Some polycations have been used to reproduce the charge-dependent effects of endogenous cationic proteins on epithelia functions in vitro.

One of these cationic polypeptides is protamine, a naturally occurring arginine-rich protein (pI 9.7 to 12) obtained commercially from salmon sperm. The molecular weight of protamine is relatively low (approximately 5,000 Da), although the chain length can vary depending on the species of fish used to obtain the polycation (Fromm et al., 1985). Several studies have shown that protamine can disturb the epithelial barrier and increase the vascular permeability in...
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rat lung (Chang et al., 1987) and brain (Olesen and Crone, 1986) as well as enhance the transport properties of alveolar and mesothelial epithelia (Alavi et al., 1982; Saumon et al., 1995). Experiments in vitro have revealed more diverse effects of protamine on epithelial functions. Thus, this polycation increases the $R_t$ across Necturus gallbladder “leaky” epithelium (Fromm et al., 1985; Bentzel et al., 1987) and cultured human and bovine ciliary epithelial cells (Straub and Wiederholt, 1991), but decreases this biophysical parameter in “tight” epithelial MDCK cells (Peterson and Gruenhaupt, 1990) and in type II pneumocytes in culture (Rochat et al., 1988). All of these studies have claimed that the protamine-induced changes in $R_t$ result predominantly from alterations in the paracellular permeability and TJ. However, the actions of this polycation on membrane permeability are controversial, and vary from claims of no effect to reports of increases or decreases in the membrane conductance to $K^+$, $Cl^-$ and $Na^+$ (Poler and Reuss, 1987; Bentzel et al., 1987; Fromm et al., 1990; Smith et al., 1997).

Such divergent effects may partly reflect the different animal species, tissues and concentration of protamine used in these studies.

In the present work, we investigated the effect of protamine on the epithelial barrier function using two strains of Madin-Darby canine kidney epithelial cells line (MDCK) that differ in TJ biochemistry and permeability properties (Barker and Simmons, 1981; Valentich, 1981; Collares-Buzato et al., 1998a, 1998b). We have demonstrated a concentration-, cell strain- and time-dependent effect of protamine on $R_t$. The exposure of MDCK II monolayers to protamine (50 µg/ml) significantly increased the $R_t$ after 10 min with a return to control values after 2 h. A higher concentration of protamine (250 µg/ml) caused an irreversible decrease in this parameter after 30 min exposure. In contrast, both concentrations of protamine markedly decreased the $R_t$ across “tight” MDCK I monolayers. Therefore, our in vitro system reproduced those various epithelial actions of protamine described in the literature using comparable

![Figure 4](image-url)

**Fig. 4.** F-actin distribution in MDCK monolayers of both strains following treatment with protamine (250 µg/ml). MDCK II and MDCK I cells were stained with TRITC-labelled phalloidin to reveal F-actin microfilaments. Monolayers of strain II (b) and strain I (d) were exposed to protamine (250 µg/ml, diluted in Krebs-Ringer buffer) for 30 min. The polycation was applied to the apical surface of the treated monolayers. The control cells (a and c) were exposed to Krebs-Ringer buffer for the same length of time. These photomicrographs are representative confocal “en face” (X-Y) images at the medium level of the monolayer and were obtained using an identical sensitivity. Note the condensation of F-actin microfilaments in some areas of the intercellular contact region in both strains of cells after treatment with protamine (arrowheads). In MDCK I cells, this alteration was less marked compared to that observed in MDCK II cells. Bar=30 µm.
doses of this polycation (Fromm et al., 1985; Straub and Wiederholt, 1991; Peterson and Gruenhaupt, 1990; Rochat et al., 1988). In accordance with previous studies (Alavi et al., 1982; Bentzel et al., 1987; Fromm et al., 1985; Peterson and Gruenhaupt, 1990), our present work also showed that alterations in transepithelial electrical resistance was observed only after apical and apical+basolateral treatment of MDCK monolayers with protamine. It has already been suggested that there may be sensitive anionic binding sites for protamine on the apical membrane that are not present on the basolateral surface of the epithelial cells (Peterson and Gruenhaupt, 1990).

The protamine-induced marked decrease in \( R_t \) was paralleled by significant increase in basal-to-apical flux of the extracellular marker phenol red. This result indicates that protamine increases paracellular permeability in both strains of MDCK cell line. However, significant increase in \( R_t \) across the low-resistance MDCK cells following protamine treatment at low concentration was not accompanied by significant changes in apical-to-basal flux of this extracellular

Fig. 5. Cellular expression and distribution of junctional proteins in MDCK II monolayers following exposure to protamine (50 μg/ml). Monolayers of strain II MDCK cells were immunostained for the tight junction-associated proteins occludin (b), claudin-1 (d) and ZO-1 (f) and then detected by indirect immunofluorescence. The treated monolayers were exposed to protamine (50 μg/ml, diluted in Krebs-Ringer buffer) at the apical surfaces, for 2 h. The control cells received Krebs-Ringer buffer alone for the same length of time (a, c and e). All of the panels are representative confocal “en face” (X-Y) images obtained at the medium level of the monolayer using an identical sensitivity. The graphics show the measurement of the fluorescence intensity of occludin and ZO-1 immunoreaction in control and treated groups. Note that protamine (50 μg/ml) induced no significant changes in the immunolabelling for these junctional proteins. Bar=30 μm.
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Occludin

Fig. 6. Cellular expression and distribution of the tight junction-associated protein occludin in monolayers of both strains of MDCK cells following treatment with protamine (250 µg/ml). The monolayers were fixed and immunostained for occludin using a standard indirect immunofluorescence protocol. Panels b and d, “en face” (X-Y) confocal images of MDCK II and MDCK I cells, respectively, showing monolayers treated with protamine (250 µg/ml) at the apical surface, for 30 min. Panels a and c, “en face” (X-Y) confocal images showing control monolayers treated with Krebs-Ringer buffer alone for the same length of time. Note that this treatment markedly decreased the junctional expression of occludin in both strains compared to the controls. In strain I, this alteration was less marked than in strain II. The graphics show the measurement of the fluorescence intensity of occludin immunoreaction in control and treated groups in both MDCK strains. The columns are the means±SE of 7 to 8 confocal images per group. All confocal images were obtained using the same sensitivity. Bar=30 µm. *P<0.05 (Student’s t-test).

marker. Possible explanations for this result are that the measurement of the transepithelial flux of phenol red may be not a method sensitive enough to detect subtle increases in paracellular permeability or, alternatively, changes in membrane conductance induced by the polycation may be contributing to the R_t measurement.

The disruption of the paracellular barrier induced by protamine was associated with a rearrangement of the cytoskeletal F-actin and impairment of the TJ integrity. We observed herein that protamine induced, in both MDCK strains, alterations in the distribution of the cytoskeletal F-actin, such as microfilament condensation in the intercellular contact region and intense staining of apical surface region, as compared to non-treated cells. The increased labeling for F-actin observed with protamine may be also indicative of actin polymerization. Similar changes in rhodamine-phalloidin staining have been described in Caco-2 cells treated with E. coli cytotoxic necrotizing factor 1 (Gerhard et al., 1998). Interestingly, in this experimental condition, the actin polymerization was also accompanied by an increased paracellular permeability (Gerhard et al., 1998).

Changes in the actin cytoskeleton have been previously described after exposure of monolayers to polycations (Peterson and Gruenhaupt, 1990; McEwan et al., 1993). In our work, we showed that the protamine-induced rearrangement of the cytoskeleton is concentration-dependent and displayed polarity being only observed following apical cell exposure to the polycation. In addition, the protamine-induced changes in cytoskeletal microfilaments appears to be one of the first events triggered by protamine-binding cell membrane since occurred even in absence of decrease of R_t and changes in paracellular permeability, as observed following 1 h treatment with a concentration of 50 µg prota-
mine/ml in MDCK II cells. Therefore, we can hypothesize that the rearrangement of the cytoskeleton may be a phenomenon that precedes the TJ disruption induced by protamine. Rearrangement of actin filaments is frequently associated with decrease in $R_t$ and alteration of the TJ structure (Collares-Buzato et al., 1994; Gerhard et al., 1998; Madara et al., 1986; McEwan et al., 1993). We do not have a complete explanation for the observation that 50 µg/ml of protamine for 1 h increased $R_t$ across MDCK II cells with reorganization of actin filaments. This data may indicate that the changes in $R_t$ may be partially attributed to changes in membrane permeability rather than exclusively to TJ permeability. Interestingly, there are reports showing that actin filament organization plays an important role in the regulation of channel activity and membrane permeability in some in vitro systems (Abdul-Ghaffar Al-Shaibani and Hagen, 2002; Negulyaev et al., 1996; Xu et al., 1997).

The protamine-induced increase in paracellular permeability and cytoskeleton redistribution were associated with marked changes in the junctional content of the TJ-associated proteins occludin in both strains and claudin-1 in MDCK II cells. Subtle alterations of immunolabelling for ZO-1 was detected following protamine treatment. To our knowledge, this is the first study to demonstrate that protamine-induced epithelial barrier disruption is associated with alterations in TJ structure and biochemistry. Occludin and claudins are transmembrane proteins forming the TJ strands and are directly involved in paracellular barrier function (Anderson, 2001; Mitic et al., 2000; Tsukita and Furuse, 2002). Mutation or overexpression of these proteins in cultured cells has been shown to affect the epithelial permeability to charged and noncharged solutes (Inai et al., 1999; Anderson, 2001). In experimental conditions, impairment of the paracellular barrier function has often been related to alterations in the junctional expression and localization of occludin and claudins (Collares-Buzato et al., 1998a, 1998b; Anderson, 2001; Tsukita and Furuse, 2002). Both occludin and claudins bind to a complex of cytoplasmic proteins (e.g. ZO-1, ZO-2, ZO-
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Claudin-1

Fig. 8. Cellular expression and distribution of the protein claudin-1 in MDCK II and MDCK I monolayers following treatment with protamine (250 µg/ml). The monolayers were fixed and immunostained for this tight junction-associated protein using a standard indirect immunofluorescence method. Panels b and d, “en face” (X-Y) confocal images of strains II and I, respectively, showing monolayers treated with protamine (250 µg/ml) at the apical surface for 30 min. Panels a and c, “en face” (X-Y) confocal images showing control monolayers treated with Krebs-Ringer buffer alone for the same length time. Note the marked decrease in the junctional expression of claudin-1 only in treated strain II MDCK monolayers, as compared to the control cells. Note also the much more punctated labelling within the cytoplasm of treated cells. The graphics show the measurement of the fluorescence intensity of claudin-1 immunoreaction in control and treated groups in both MDCK strains. The columns are the means±SE of 4 to 7 confocal images per group. All confocal images were obtained using the same sensitivity. Bar=30 µm. *P<0.05 (Student’s t-test).

3, cingulin, 7H6 antigen, symplekin and others) that have been identified in the cytoplasmic submembraneous plaque underlying membrane contacts (Fanning et al., 1998; Itoh et al., 1999; Schneeberger and Lynch, 2004). These proteins appear to organize the occludin and claudins within the TJ region and couple them to actin microfilaments (Anderson, 2001; Schneeberger and Lynch, 2004). Interactions between TJ-associated proteins and the cytoskeleton play a pivotal role in the regulation of paracellular permeability (Madara et al., 1986; Madara, 1998). Thus, after interacting with anionic sites of the apical membrane and triggering a still unknown intracellular signaling system, protamine elicits a disorganization/polymerization of cytoskeletal microfilaments. Because of this change in the cytoskeleton structure, the tension of the perijunctional actin ring may increase and/or the interaction with the TJ protein complex may be impaired, thereby resulting in disruption of the paracellular barrier. As a result, the whole TJ complex is disassembled and the proteins are internalized. Alternatively, the protamine may affect the TJ structure and function directly by binding to anionic sites lining the aqueous pores of the TJ (Anderson, 2001), whose access may be facilitated by apical cell exposure to this polycation.

Another finding of this work was that the two MDCK strains responded differently to protamine treatment. In strain I “tight” monolayers, protamine (50 and 250 µg/ml) decreased the $R_t$ values in association with an enhanced basal-to-apical phenol-red flux while, in strain II cells, changes in $R_t$ were dependent of protamine concentration. Using morphological methods, MDCK I cells showed similar changes in the localization and expression of the junctional proteins and F-actin when compared with strain II “leaky” MDCK cells. However, the protamine-induced alterations in the cytoskeleton and TJ were less intense in
MDCK I cells compared to MDCK II cells. This assumption is further strengthened by the observation described herein that 250 µg protamine/ml induced significant downregulation of occludin and claudin-1 in MDCK II but only of occludin in MDCK I. Since the claudin family is known to constitute the backbone of TJs, this result suggests a more severe disruption of TJ in MDCK II than in MDCK I cells, induced by this cationic agent. The differences in response to protamine between MDCK I and II cells reported here may related to differences in the type of intracellular signaling triggered by this polycation or to the nature and amount of anionic cell sites for protamine interaction. Interestingly, it has been shown that MDCK I cells release significantly higher amounts of anionic macromolecules, such as heparin sulfate proteoglycans, to the apical medium than MDCK II cells (Svennevig et al., 1995). These anionic molecules could potentially bind to protamine, limiting its interaction to the MDCK I cells.

In conclusion, treatment with protamine in vitro induced diverse effects on the epithelial barrier function of the MDCK cells. The changes in $R_t$ and transepithelial flux of an extracellular marker were dependent on the length of exposure to protamine, on the polycation concentration employed, and on the MDCK strain. Our results agreed with data reported in the literature on the divergent effects of protamine on epithelial function. Thus, our in vitro system using the two strains of MDCK cells provides a good model for investigating the epithelial action of polycations. The protamine-induced disruption of the epithelial paracellular barrier was associated with the rearrangement of F-actin and with a marked decrease in the junctional immunoreaction for TJ-associated proteins. These results suggest that both the cytoskeleton and TJ proteins are possible cellular targets for the protamine.

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