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Effect of FCCP on tight junction permeability and cellular distribution of ZO-1 protein in epithelial (MDCK) cells

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The effect of the uncoupler of oxidative phosphorylation, FCCP (carbonylcyanide *p*-trifluoromethoxyphenylhydrazone), on the tight junction of Madin-Darby canine kidney cells was examined. FCCP induced an abrupt decrease in the transepithelial electrical resistance of the confluent monolayers over a period of 20 s. When FCCP was withdrawn from the incubation medium, the monolayer resistance recovered to close to the original level in less than 2 h. Staining of the tight junction-associated protein ZO-1 showed that the changes in transepithelial electrical resistance were accompanied by a diffusing of the protein away from cell peripheries and a reconcentration to the tight junction areas following resistance recovery. Intracellular pH was decreased by FCCP on a similar time-scale with no obvious changes in ATP levels over this time-course. These data suggest that the uncoupler FCCP has a profound effect on tight junction permeability and cellular distribution of the tight junction protein ZO-1 in the epithelial cells and that it probably acts by breaking down proton gradients and altering intracellular pH.

The Madin-Darby canine kidney (MDCK) cell represents a cell line of cultured epithelium. Transepithelial electrical resistance develops in confluent monolayers by virtue of the formation of tight junctions intercellularly [1,2]. The tight junction is a dynamic structure which has been shown to be regulated in MDCK and other types of epithelial cell by many factors. For example, cytoskeleton-active drugs [3–6], cyclic AMP [7], protein kinase C [8], and calcium ion [2] have all been shown to alter transepithelial electrical resistance. The cytoskeleton has been thought to play a key role in the control of the tight junction [4,5]. The protein ZO-1 is a well-characterized protein which has close association with tight junctions in a variety of epithelial and endothelial cells [9,10]. In this study, we examined the effect of the uncoupler of oxidative phosphorylation carbonylcyanide p-(trifluoromethoxy)phenylhydrazone (FCCP) on the MDCK monolayer resistance, an indicator of tight junction dynamics, and on the cellular staining pattern of the protein ZO-1.

For the measurement of transepithelial resistance, MDCK cells were seeded at high density, $1 \cdot 10^6$ cells/cm^2, onto porous filter inserts (24.5 mm diameter and 0.4 μm pore size; Nucleopore). Cells were allowed to grow for 24 h in a mixture of 50% Dulbecco's modified Eagle's medium (DMEM) and 50% medium F-12 with 10% bovine serum (FCS) (all from GIBCO) and 1% penicillin-streptomycin (Sigma) under an atmosphere of 5% CO_2 and 95% air. Such monolayer preparations could develop net resistance of 350 to 500 $\Omega \cdot$ cm^2. A Millicell-ERS apparatus (Millipore) was used to measure the transepithelial electrical resistance. Fig. 1 shows the effect of varied concentrations of FCCP on the resistance. FCCP was dissolved in a small volume of dimethyl sulfoxide (DMSO) and added to the apical side of the monolayers only. FCCP caused a rapid drop in the resistance in a dose-related manner. Steady state was reached in less than 1 min.

After the treatment with FCCP, cells in monolayers were examined for viability. Trypan-blue exclusion indicated that there was no viability change after the treatment with 2 μM FCCP for 1 min, the condition used in both Figs. 2 and 3. Fig. 2 shows the recovery of transepithelial resistance following the incubation with FCCP. The resistance almost completely recovered in a matter of 1 h. The time-course is similar to that for the resistance recovery following calcium ion depletion [1], suggesting a reformation of disrupted tight junctions.

An examination of the distribution of the ZO-1 protein was carried out following a modification of the procedures described by Stevenson et al. [9]. Confluent MDCK monolayers were prepared on the culture cham-

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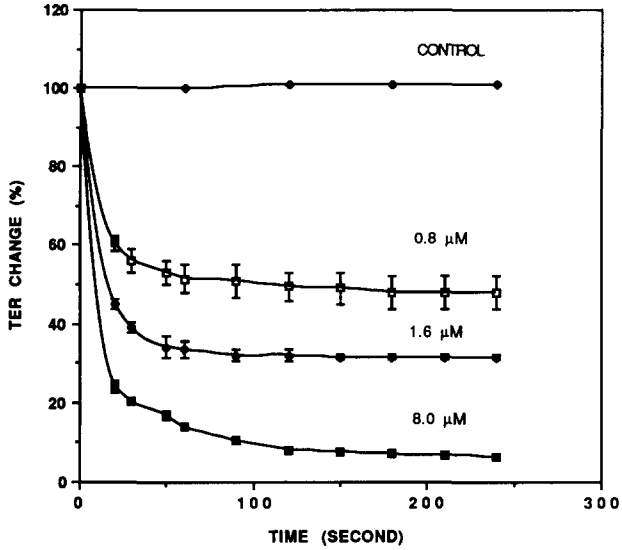

Fig. 1. The effect of FCCP on the transepithelial electrical resistance (TER) of confluent MDCK monolayers. The concentrations of FCCP in μM used are indicated. Percentage changes in resistance for each individual sample was calculated against its own starting resistance level before the average of the values from three samples for each curve were obtained. The experiments were done in the medium DMEM/F-12 (50%50%) plus 10% FCS. DMSO was also added to the control.

ber/slides (Nunc, Neperville, IL), which were coated with 100 μg/ml polylysine (Sigma) in the same way as on the filter inserts. 24-h-old monolayers were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS; GIBCO) for 10 min. The permeabilized cells were stained for the protein ZO-1 by the following incubations: (1) anti-ZO-1 monoclonal antibody R40.76 (a gift from Dr. B.R. Stevenson in Department of Anatomy and Cell Biology of the University of Alberta) contained in the DMEM with 10% FCS for 1–2 h; (2) a second antibody biotinylated rabbit anti-rat IgG (Vector, CA), 7.5 μg/ml in DMEM plus 10% FCS for 1–2 h; (3) affinity fluorescence label Texas red conjugated streptavidin (Amersham), 4 μl/ml in PBS for 1 h. Visualization and photography of the protein ZO-1 fluorescence staining were done using a Zeiss ICM 405 fluorescence microscope. Fig. 3 shows the FCCP effect on cellular distribution of the protein ZO-1 by immunofluorescence labelling and on monolayer continuity by phase contrast microscopy in MDCK cells. As shown previously [9,10], ZO-1 protein is exclusively localized to junctional areas or cell peripheries (Fig. 3a). Upon FCCP treatment, most of the protein was redistributed from the junctional areas to the cytoplasmic areas (Fig. 3b). There were corresponding changes in monolayer continuity by phase contrast microscopy (Fig. 3d and 3e). Intercellular spaces became wider. After the treated monolayers were incubated in FCCP-free medium for 1.5 h, they returned to the pretreatment state both in the cellular distribution of ZO-1 (Fig. 3c) and in monolayer morphology (Fig. 3f).

These data suggest that FCCP can reversibly open the tight junctions in MDCK cells. Our initial intention was to examine the effect of energy on tight junction permeability based on the observation in intestinal epithelium by Pappenheimer [11] that transportable nutrients, glucose and amino acids, can increase paracellular or tight junction permeability through increasing intracellular energy levels. FCCP is known as an uncoupler of oxidative phosphorylation [12]. It interrupts the coupling between the proton gradient and ATP synthesis. We intended to use FCCP here as an energy depleter. To our surprise, an examination of intracellular ATP concentration by high-performance liquid chromatography [13] showed that 8 μM FCCP did not cause a significant decrease in the ATP concentration in MDCK cells within 4 min, during which time FCCP caused significant changes in transepithelial electrical resistance, ZO-1 cellular distribution and cell morphology. In the presence of FCCP, the intracellular ATP concentration might be maintained by compensatory mechanisms, especially by increased energy production through glycolysis [14] or it might take longer for ATP stores to begin to be depleted.

Another consequence of transmembrane proton gradient disturbance by FCCP is a change in intracellular pH [15]. Measurements showed that FCCP at the concentrations affecting tight junction permeability induced intracellular pH drops (Fig. 4). It has been

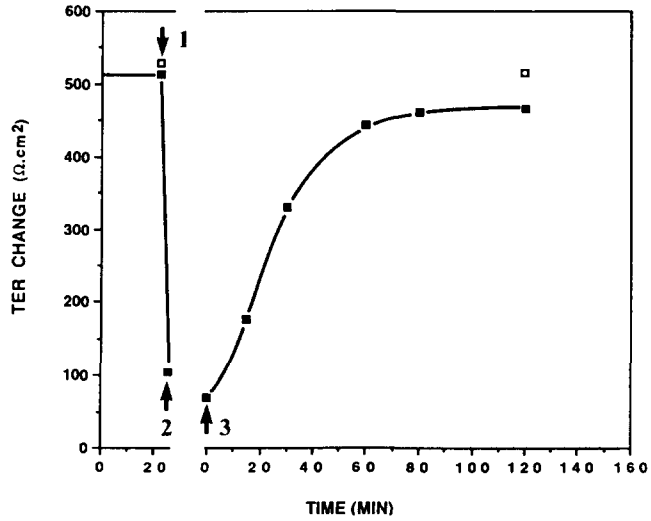

Fig. 2. Post-FCCP recovery of MDCK monolayer resistance. The cells were first incubated with 2 μM FCCP for 1 min in DMEM/F-12 (50%50%) with 10% FCS, then washed with PBS twice, and changed to FCCP-free medium (the same medium as the above) to examine the time course of resistance recovery. Arrows indicate the addition of FCCP (1), the PBS washes (2) and the change to FCCP-free medium (3). Control samples (open squares) were subject to the same handling as the test samples (solid squares). The standard errors were less than 10% ($n = 3$).

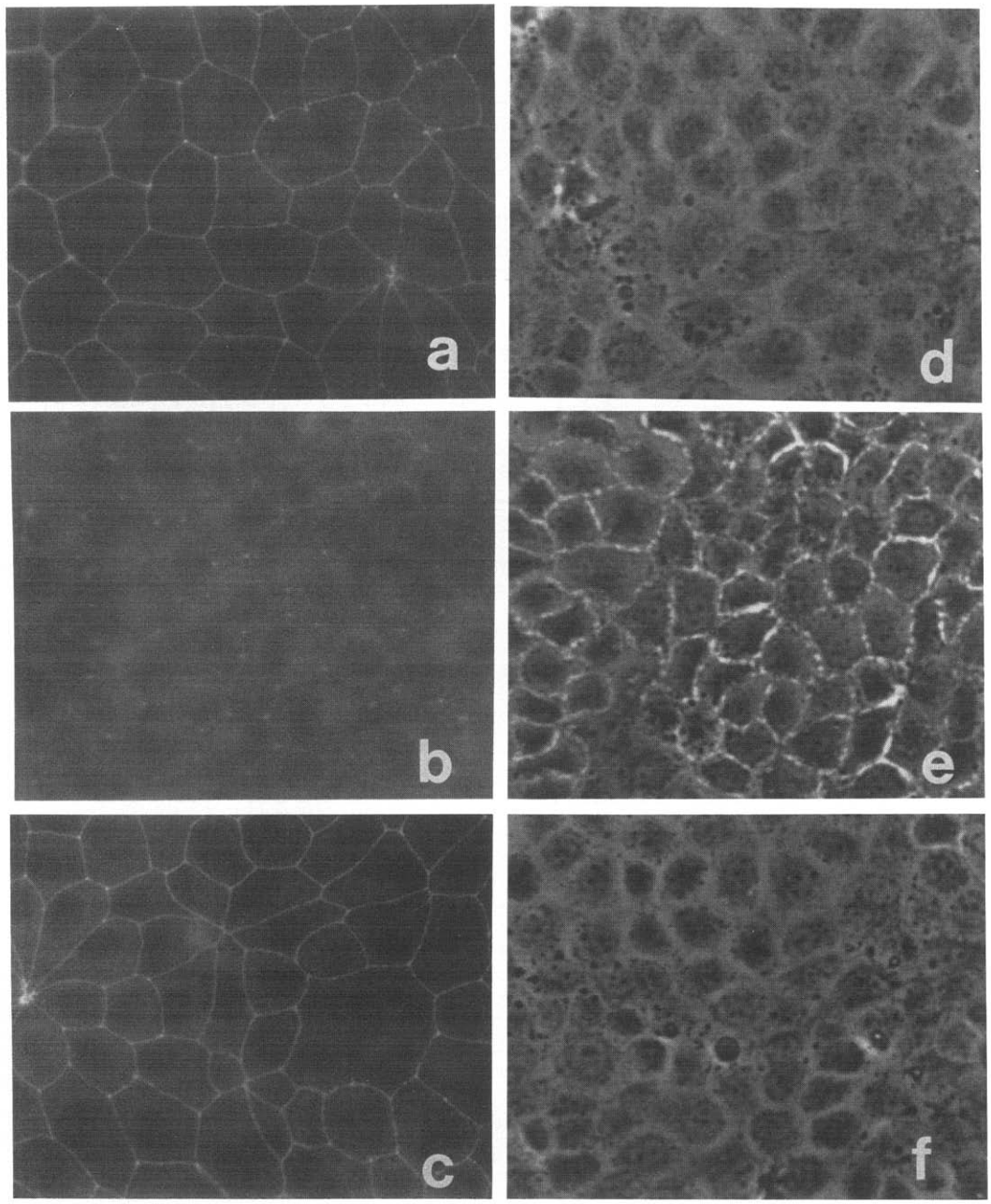

Fig. 3. The effect of FCCP treatment on the distribution of the protein ZO-1 in MDCK monolayers. Immunofluorescence photomicrographs (a, b, c) are shown on the left side and the corresponding phase contrast microscopy of the cell monolayers (d, e, f) are shown on the right side. a and d were not treated with FCCP. b and e were treated with the drug for 1 min at a concentration of 2 μM. c and f were treated with FCCP the same way as b and e first, then transferred to FCCP-free medium (see Fig. 2) and incubated for an additional 1.5 h. The cells were then fixed for immunofluorescence labelling of the protein ZO-1 and phase contrast microscopy.

shown that FCCP caused the complete disruption of cellular microtubules in BHK21 cells by affecting intracellular pH [16]. By the same token, FCCP could profoundly affect cytoskeleton or tight junction assembly. Comparing the time-courses for the resistance changes (Fig. 1) and intracellular pH changes (Fig. 4), a close similarity is observed, suggesting that an intracellular pH decrease could be directly responsible for the increase in tight junction permeability. It has previously been demonstrated that calcium ion chelation [15,16] also results in the interruption of tight junction integrity, decreased transepithelial resistance and a redistribution of the ZO-1 protein from the cell periphery to the cytoplasm. FCCP caused a similar change in our preparation (Fig. 3b) which occurred in less than 1 min.

We put forward the following hypothesis. FCCP, in acting to break down proton gradients, alters the intracellular pH which results in an alteration of the forces,

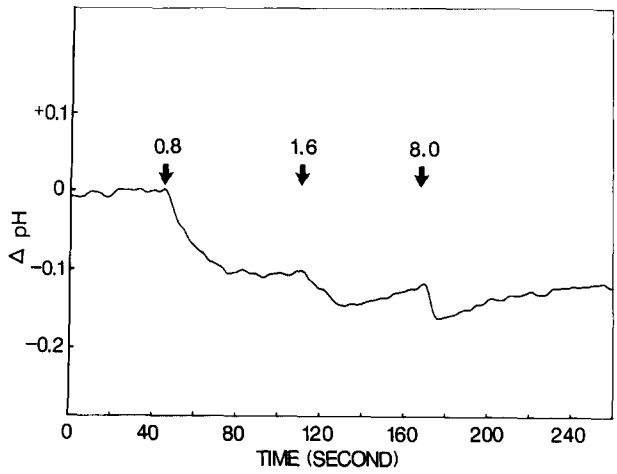

Fig. 4. Effect of FCCP on intracellular pH in MDCK cells. Cells in confluent monolayers were trypsinized (0.04% trypsin in calcium-free Puck's saline with EDTA) removed from the culture flasks, washed with PBS and then incubated with a fluorescent pH indicator, BCECF/AM (Molecular Probes), at 2 μM in PBS for 1 h at 37°C [19]. The cells were then washed three times with PBS and resuspended in the buffer at a density of $2 \cdot 10^6$ cells/ml for intracellular pH measurement using a fluorescence spectrophotometer (SLM-8000C, AMINCO) with a thermostatically controlled cell holder fitted with a magnetic stirrer. The numbers indicate the accumulative drug concentrations in μM. The solvent DMSO, at the same concentrations, did not cause any change in intracellular pH.

possibly electrostatic, which keep the ZO-1 protein at the region of the tight junction. There is now a strong correlation between the ZO-1 protein, the integrity of the tight junction and the transepithelial electrical resistance. When changes in electrostatic or other pH-dependent forces occur, there is a dissociation of the ZO-1 protein from the region of the tight junction, the transepithelial resistance decreases sharply and the ZO-1 proteins are seen to move from the tight junction band. Removing the FCCP allows the pH gradients to reform, but this is a much slower process and it takes up to an hour for the pH to return to normal, for the ZO-1 protein to reestablish itself as a tight junction protein and for the subsequent transepithelial resistance to increase to its normal level. Obviously, this hypothesis requires further testing, especially to link the changes in resistance directly to the dissociation of the ZO-1 protein as a primary event rather than a direct effect of the pH change on the transepithelial electrical resistance and a subsequent disruption of the tight junction.

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