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Determination of the Na Permeability of the Tight Junctions of MDCK Cells by Fluorescence Microscopy

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Abstract. The kinetics of Na movement across the tight junctions of MDCK cells, grown on coverslips and perfused with HEPES or bicarbonate Ringer at 37°C, were investigated after filling the lateral intercellular spaces (LIS) of the epithelium with SBFO, an Na-sensitive fluorescent dye. Dilution and bi-ionic potential measurements showed that MDCK cell tight junctions, although cation-selective, were poorly permeable to N-methyl-D-glucamine Cl (NMDG) but freely permeable to Li. In previous experiments in which Na was replaced by NMDG, a very slow decrease in LIS Na concentration (time constant = 4.8 min) resulted. In the present study, reduction of perfusate Na from 142 to 14 or 24 mM with Na replaced by Li caused LIS Na concentration to decrease with a time constant of 0.43 min. The time constant for Na increase of the LIS was 0.28 min, significantly shorter than that for Na decrease because of the additional component of transcellular Na influx. Ouabain eliminated the transcellular component and equalized the time constants for Na influx and efflux. These results were incorporated into a mathematical model which enabled calculation of the transcellular and paracellular Na fluxes during fluid reabsorption. Regulation of the Na permeability of individual tight junctions by protein kinase A (PKA) was evaluated by treating the monolayers with the Sp-cAMPS, a cAMP substitute, or Rp-cAMPS, a specific inhibitor of PKA. Stimulation of PKA strikingly increased tight junctional permeability while PKA inhibition diminished junctional Na permeability.

Key words: Na — cAMP — SBFO — Protein kinase A — Transference number

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Introduction

Delineation of the permeability properties of epithelial tight junctions (TJ) is made difficult because of the influence of the fluid-filled lateral intercellular spaces (LIS) adjoining the TJ (Kottra & Frömter, 1993*a,b*). In previous investigations of the composition of the LIS of Madin-Darby canine kidney cells (MDCK), the relative Na and Cl permeabilities of the TJ were determined from the rate constants for changes in LIS ion concentration (Chatton & Spring, 1995; Xia, Persson & Spring, 1995). The measurements of Na concentration in the LIS of MDCK cells revealed a long LIS Na time constant (4.8 min) compared to that of Cl (0.8 min). This result seemed inconsistent with previously published electrophysiologic data (Oberleithner et al., 1990*a,b*) in which the transference number of the tissue for Na was 0.64, about twice as high as that for Cl. It was speculated by Chatton & Spring (1995) that the slow Na time constant arose not from a limited permeability of the TJ to Na but from a limited permeability to N-methyl-D-glucamine (NMDG), the Na substitute employed in that study. Two possible explanations for the discrepancy between the optical and electrical data were considered: (i) the MDCK cells used by Chatton and Spring (1995) differed in TJ permeability properties from those used by other investigators; (ii) the previously measured rate constant for Na was in error because of the use of an impermeant Na substitute.

In the present investigation, the electrophysiologic properties of MDCK cell layers were assessed by measurements of bi-ionic and dilution potentials, calculations were conducted to determine parameters which were compatible with both the optical and electrical data of present and previous studies, the Na time constant was determined with Li as a Na substitute. Li was selected

on the basis of the electrophysiologic measurements as a replacement for Na with equal permeability across the paracellular pathway. Finally, the regulation of the permeability of the TJ by intracellular cAMP was evaluated by measurement of LIS Na and fluorescent dye concentration when the monolayers were exposed to analogues of cAMP.

Materials and Methods

Cell Culture

Low resistance MDCK cells, passage 66–79 from the American Type Culture Collection (Rockville, MD) were cultured as previously described (Harris et al., 1994), using Dulbecco's modified Eagle medium (DMEM) and 2 mM glutamine without added riboflavin, antibiotics and phenol red. The culture medium for stock cells was supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY). For the experiments, the cells were seeded on glass coverslips and cultured for 5–11 days.

For some of the electrophysiologic studies, cells were grown on 24 mm Anocell membranes in DMEM supplemented with 1% fetal bovine serum as previously described (Chatton & Spring, 1994).

Experimental Solutions and Perfusion System

HEPES-buffered experimental solutions contained (mM): 142 Na^+, 5.3 K^+, 1.8 Ca^{2+}, 0.8 Mg^{2+}, 137 Cl^-, 0.8 SO_4^{2-}, 14 HEPES, 5.6 glucose. The pH of the HEPES solutions was adjusted to pH 7.4 at 37°C and were gassed with room air. The bicarbonate-buffered solutions contained (mM): 142 Na^+, 5.3 K^+, 1.8 Ca^{2+}, 0.8 Mg^{2+}, 127 Cl^-, 0.8 SO_4^{2-}, 24 HCO_3^-, 5.6 glucose, and were gassed with 5% CO_2/95% air.

Four low Na solutions were used—the HEPES-buffered solution contained (in mm): 14 NaCl and 128 LiCl or 128 NMDGCl while the bicarbonate-buffered solution contained 24 NaCl and 118 LiCl or 118 NMDGCl. The osmolarity of all solutions was 292–300 mOsm/kg. The cell monolayers were perfused in a closed chamber designed for rapid solution exchange (Harris et al., 1994). The perfusion solutions were kept aerated at 37°C using water-jacketed reservoirs permitting temperature control and gas mixing. The lines coming from the different reservoirs could be opened and closed using computer-controlled pinch-valves and were connected to a manifold.

Chemicals

SBFO (ammonium salt) was obtained from Molecular Probes (Eugene, OR). Ouabain octahydrate was purchased from Sigma (St. Louis, MO) and weighed just before each experiment. (S_p)-Adenosine cyclic 3′,5′-phosophorothioate (S_p-cAMPS) and (R_p)-Adenosine cyclic 3′,5′-phosphorothioate (R_p-cAMPS) were obtained from Biolog (La Jolla, CA).

Fluorescence Microscopy

The experiments were performed on the stage of an upright microscope (Ortholux II, Leica, Deerfield, IL) equipped for bright field and low light level fluorescence (Chatton & Spring, 1993). Epifluorescence illumination was achieved using a dual path 75W xenon lamp assembly (Model 60000, Oriel, Stratford, CT) equipped with 10 nm bandpass 340 nm and 380 nm filters (Omega Optical, Brattleboro, VT). Switching the wavelength between 340 nm and 380 nm was done by opening and closing fast shutters (Uniblitz, Vincent Associates, Rochester, NY). The light source was connected to the microscope by means of a fused silica optical fiber (C Technologies, Verona, NJ). The fluorescence filter cube contained a 400 nm dichroic mirror and a 430 nm barrier filter. Bright field imaging was achieved with a 50W tungsten halogen lamp (Leica).

The cell monolayers were observed with a 100×/1.3 N.A. objective lens (Nikon, Melville, NY) connected to a microchannel plate intensifier (KS-1381, Video Scope, Sterling, VA) and video camera (VS-2000N, Video Scope). An 8-frame running average was used to reduce the noise level of the image (LKH 9000, Video Scope) which was stored on an optical memory disc recorder (OMDR, TQ-2028F, Panasonic, Newark, NJ) for later offline analysis. The sequence of events (e.g., solution valves, intensifier gain, illumination shutters, stepper motor) during the experiment was controlled by a computer using a custom-made program.

SBFO Loading into the LIS

SBFO was incorporated into the LIS, as previously described (Chatton & Spring, 1995). Briefly, the cell monolayers were incubated in their culture dish for 70–80 min with the free acid SBFO (~250 μM in buffered solution with 142 mM Na). During the incubation period, the dye molecules passively diffused across the tight junctions and progressively filled the LIS compartment and the domes. After washout of the fluorescent dye from the bathing solution, the SBFO trapped in the LIS allowed measurements for up to 60 min until the signal-to-noise ratio diminished because of back diffusion of the dye through the tight junctions.

Analysis of Fluorescence Microscopy Images

Images were transferred from the optical disc recorder to an image analysis workstation (Trapix 55/48Q, Recognition Concepts, Incline Village, NV) as was described previously (Chatton & Spring, 1995). Briefly, segmentation of the bright LIS and dark cellular regions was obtained from a binary image after thresholding of the 380 nm image. Regions of the LIS separated by small intensity difference were connected to each other by repeated dilation and erosion of the binary image, and the outline of the LIS was used as a template for both 340 nm and 380 nm images. A ratio of the mean pixel intensity inside the template of the two images was used as a measurement of Na concentration.

Differential Interference Contrast Microscopy

High resolution images of the cells and adjacent LIS were obtained by the use of differential interference contrast (DIC) imaging of monolayers grown on glass coverslips and perfused at 37°C on the stage of an inverted microscope (IX70, Olympus, Lake Success, NY). Cells were imaged with a 100×/1.4 N.A. objective lens, further magnified 1.8-fold, and projected onto the faceplate of a CCD camera (CCD 200, Video Scope), stored on optical disc (TQ-2025F, Panasonic), and analyzed by a program designed to automatically detect cell boundaries and to measure cell size and shape (Marsh et al., 1985). Optical sections of MDCK cells and adjacent LIS were obtained at 0.4 μm focus displacements.

Cell volume was calculated from the product of the measured areas of the optical sections and the known focus displacements as previously described (Marsh, Jensen & Spring, 1985). LIS volume was

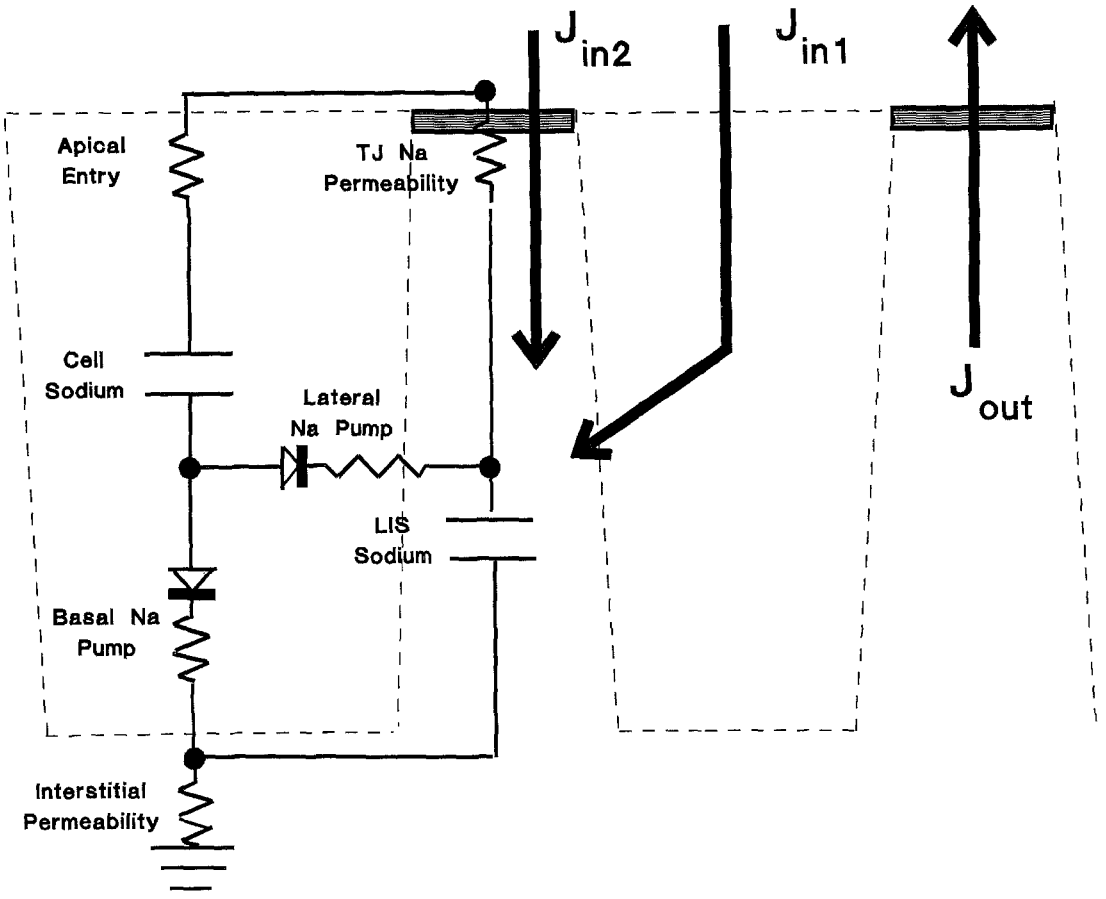

Fig. 1. On the left side, the model for sodium transport in the MDCK cell monolayer is schematically represented in the form of the electrical equivalent circuit. The Na capacity of the cell and LIS are depicted by capacitors whose values are determined from the product of volume of each compartment and their respective Na concentrations. Diodes in series with resistors represent the Na,K-ATPase which results in a Na flux in only one direction — from cell to LIS. The fluxes of Na from bath to cell and LIS are denoted as J_{in1} and J_{in2} respectively; Na flux from LIS to bath is denoted as J_{out}.

determined from the difference in volume of the cell plus LIS and the cell alone.

Electrical Measurements

Transepithelial dilution potential measurements were made at 25°C on monolayers grown on glass coverslips by micropuncture of a fluid-filled dome with a glass microelectrode filled with 3M KCl. The perfusate composition was switched from control HEPES-buffered Ringer to a low NaCl solution in which half of the NaCl was replaced by sucrose. The microelectrode was connected through a Ag-AgCl electrode to the probe input of an electrometer (Model 750, World Precision Instruments, Sarasota, FL). The apical bath was grounded through a Ringer-filled agar bridge to a calomel electrode immersed in a saturated KCl bath. Stability criteria for the electrode input resistance and tip potential were as previously described (Fisher & Spring, 1984).

Transepithelial dilution and bi-ionic potentials were also measured at 25°C with similar solutions across monolayers grown on permeable supports and mounted in an Ussing chamber (Model Milli-24/2, World Precision Instruments, Sarasota, FL). Potential and current electrodes were Ag-AgCl electrodes connected to the chamber by 3M KCl agar bridges. The reference electrode was placed in the basolateral bath. Transepithelial voltages and resistance were measured with a high impedance electrometer connected to the relevant electrodes.

Mathematical Model

Figure 1 shows the electrical equivalent circuit model of the epithelium employed to simulate the response of the monolayer to alterations in the Na concentration of the apical bathing solutions. Calculations were carried out on a PC using the SPICE simulation program (Intusoft, San Pedro, CA). Initial parameter values were selected based on published data on the electrical resistance and ionic selectivity of the cell membranes and MDCK cell monolayers. Capacitance values in Fig. 1 correspond to the size of the cellular and LIS compartments multiplied by their respective Na concentrations. The Na,K-ATPase was represented by a diode and resistor in series to simulate the unidirectional flow of Na from cell to the LIS.

Statistics

Data are presented as means ± SE. Statistical significance was determined using the paired or unpaired t test and a P value < 0.05 was considered significant.

Table 1. Bi-ionic and dilution potentials of MDCK monolayers

Condition	Ion substitution	PD (mV)	*n*
14 mM Na-apical 142 mM Na-basolateral	NMDG	25.0 ± 6.5	5
14 mM Na-apical 142 mM Na-Basolateral	Li	1.3 ± 0.1	4
50% Dilution-apical 142 mM NMDG-Cl basolateral	NMDG	0.5 ± 2.6	5
50% dilution-apical 142 mM LiCl basolateral	Li	11.4 ± 1.7	4

Experiments were performed on cells grown on permeable supports and studied in an Ussing chamber. Bathing solutions were buffered with HEPES.

Results

Dilution Potential Measurements

The transepithelial NaCl dilution potential (HEPES buffer) measured by micropuncture of the domes of monolayers averaged 10.3 ± 1.2 mV (15 punctures in 7 monolayers), dome interior negative, when the apical bath NaCl concentration was reduced by 50%. Such an NaCl dilution potential is consistent with a cation-selective tight junction with a Na transference number of 0.82. A similar result was obtained with cells grown on the permeable supports. The transepithelial dilution potential averaged 11.1 ± 1.2 mV (8 monolayers), basolateral solution negative, when the apical bath was diluted by 50%. When the basolateral bath was similarly diluted, the transepithelial potential difference (PD) was −11.4 ± 2.1 mV (4 monolayers), apical bath negative.

Symmetry of dilution potentials and lack of rectification of the transepithelial PD are indicative of the dominance of a paracellular pathway as the site of transepithelial ion selectivity (Barry & Diamond, 1984). The dilution potentials observed were less than that expected for a perfect Na electrode, 16.1 mV at 25°C with a ratio of NaCl activities of 1.9, but were consistent with a cation-selective tight junction. The transference number for Na calculated from the dilution potentials across cells grown on filters ranged from 0.84 to 0.85, slightly higher than the value for MDCK cell monolayers of 0.64 reported by previous investigators (Oberleithner et al., 1990*a,b*).

Bi-ionic Potential Measurements

We next measured bi-ionic potentials across monolayers grown on permeable supports to learn more about the cation selectivity of the tight junctions. Table 1 shows the bi-ionic potential across MDCK cell monolayers in which all but 14 mM of the Na in the apical perfusate (HEPES buffer) were replaced by NMDG or Li. Replacement by NMDG resulted in a transepithelial PD of 25.0 ± 6.5 mV, apical bath positive. This is consistent with a low permeability of the tight junction to NMDG compared to that of Na, a conclusion confirmed by a 3.7-fold increase in transepithelial electrical resistance when all but 14 mM Na in both bathing solutions were replaced by NMDG. Further confirmation of the low tight junctional permeability to NMDG came from determination of the transepithelial dilution potential in NMDG Ringer when the apical bath NMDG concentration was reduced to 50% of that in the basolateral bath. The transepithelial PD was 0.5 ± 2.6 mV, indicative of insignificant NMDG permeation, and consistent with the conclusion that junctional permeability to NMDG was very low.

When the all but 14 mM of the Na in the apical perfusate was replaced by Li the transepithelial PD was only 1.3 ± 0.1 mV, apical side positive, consistent with the conclusion that the tight junctions do not discriminate between Na and Li (Table 1). In support of this conclusion, the transepithelial resistance of monolayers bathed in symmetrical LiCl Ringer solutions was indistinguishable from that in NaCl Ringer. Finally, the potential generated by a 50% dilution of the apical bath was 11.4 ± 1.7 mV, apical side positive, in LiCl Ringer solutions, comparable to that obtained in NaCl Ringer (Table 1).

The electrophysiologic experiments showed that our MDCK cells were cation selective as had been reported previously by others (Oberleithner et al., 1990*a,b*) and that the permeability of the tight junctions to NMDG was low compared to that of Na. Thus, previous experiments employing NMDG as an Na substitute in the apical perfusate must have resulted in a large bi-ionic potential across the tight junction which impeded Na efflux from the LIS. It was also evident from these experiments that Li replacement of Na had none of these undesirable effects. We, therefore, repeated previous experiments to determine the rate of change of Na in the LIS using Li as an Na substitute.

Kinetics of Na Changes in LIS

In the first series of experiments, the kinetics of Na changes in LIS of MDCK cells grown on glass coverslips were determined in HEPES and bicarbonate solutions. Monolayers were perfused on the apical surface with solutions containing 142 mM Na. After a control period, the perfusate Na concentration was rapidly switched from 142 mM to the low Na (14 mM or 24 mM) solution. After the LIS Na concentration achieved a new steady-state, the perfusion solution was changed back to the high Na concentration. Na was replaced isosmotically by Li; the Cl concentration was unchanged. Acquisition

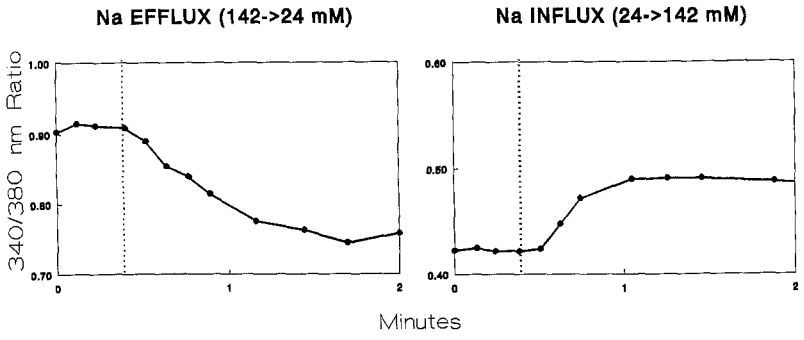

Fig. 2. Typical experimental results for the kinetics of Na concentration changes in the LIS. Vertical lines correspond to the times of changes in the Na concentration of the apical perfusate.

of images was performed every few seconds over 10 min at one focal plane midway between the top and bottom of the LIS.

A typical example of the time course of Na changes in LIS is illustrated in Fig. 2. The time constants for Na efflux from LIS and influx into the LIS were obtained by nonlinear curve fitting using the following equation:

$$R = A + B \cdot e^{\{-t/\tau\}} \quad (1)$$

where R is the 340/380 nm ratio, t is the time, τ is the characteristic time, A and B are optimized parameters.

The flux of Na into or out of the LIS (J) was calculated from the rate constant, k, equal to the $1/\tau$ in Eq. 1, the measured LIS Na concentration, C_{LIS}, the bathing solution Na concentration, C_{Bath}, and the LIS volume of one cell, V_{LIS}.

$$J = k\ (C_{Bath} - C_{LIS})\ V_{LIS} \quad (2)$$

In Eq. 2, k represents the permeability coefficient of the tight junction for a cell whose LIS has a volume, V_{LIS}, of 46.5 fl.

Figure 3 shows the Na efflux from the LIS in HEPES solutions for 9 monolayers calculated from Eq. 2 with the measured time constant of 0.48 ± 0.03 min and an estimated transjunctional Na concentration difference of 128 mM. As shown in Table 2, the Na efflux was significantly larger than the Na influx calculated from the time constant of 0.66 ± 0.07 min and a similar transjunctional concentration gradient.

Figure 3 also shows that in bicarbonate-buffered solutions the calculated Na efflux was similar to that in HEPES based on a time constant of 0.43 ± 0.02 min and a Na concentration gradient of 133 mM. As predicted by the mathematical model, Na influx was significantly larger than efflux calculated from the influx time constant of 0.28 ± 0.03 min and a 118 mM Na gradient across the tight junction (Table 2).

Na influx in bicarbonate-buffered solutions was significantly ($P < 0.01$) greater than that observed in HEPES buffer, an observation in agreement with the conclusions from previous measurements of LIS Na concentration that Na transport is stimulated in bicarbonate-buffered solutions (Chatton & Spring, 1995).

Mathematical Model

The LIS Na transients were analyzed using a model in which there are two different pathways for Na influx (J_{in}) into the LIS of MDCK cells (Fig. 1): (i) the transcellular route (J_{in1}) which involves Na entry via the apical membrane and exit from the cell across the basolateral membrane mediated by the Na,K-ATPase; (ii) direct Na influx across the *TJ* (J_{in2}). The model assumes that there is only one Na efflux pathway from the LIS to apical bath-Na flux through the *TJ* (J_{out}).

The model calculation predicts that changes of Na concentration in apical solution result in an Na influx into the LIS which is faster than efflux because there are two influx pathways and one efflux pathway. The addition of ouabain to inhibit the transcellular flux of Na (J_{in1}) would be predicted to equalize influx and efflux, assuming that the tight junctions do not rectify the flux of Na across them. The model calculations were based on the assumption that the volume of the cell and LIS are constant. We were concerned that this assumption may be incorrect when the monolayers were treated with ouabain to block transcellular Na transport. Accordingly, a series of experiments were undertaken to assess the effects of ouabain on cell and LIS volume.

Effect of Ouabain on Cell and LIS Volume

Preliminary experiments showed, in agreement with previous studies (Simmons, 1981; Chatton & Spring, 1995), that ouabain produced complete inhibition of the Na,K-ATPase of MDCK cells within 10 min. Therefore, after the control period in which optical sections at focus displacements of 0.4 μm of selected cells and their adjacent LIS were obtained in bicarbonate-buffered solution, the perfusate was switched to a solution of the same composition (142 mM Na) containing 5×10^{-4} M ouabain. After an equilibration time of at least 10 min, high res-

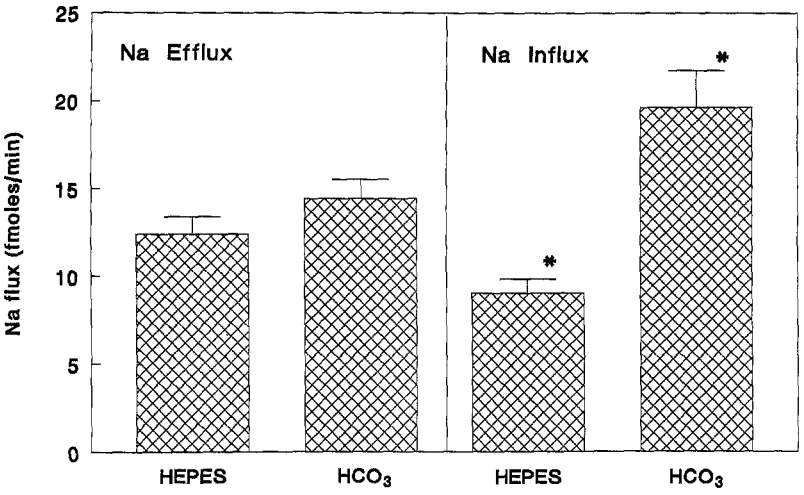

Fig. 3. Mean values with SEM of sodium efflux from the LIS and influx into the LIS in HEPES and bicarbonate/CO_2 solutions. Na efflux in HEPES is significantly higher than influx ($P < 0.05$) while Na efflux in the presence of bicarbonate/CO_2 is lower than influx ($P < 0.05$). The number of experiments was 9 in all groups. Efflux does not differ significantly in HEPES or bicarbonate/CO_2 while influx in the presence of bicarbonate/CO_2 is larger ($P < 0.001$) than that in HEPES.

Table 2. Time constants and corresponding Na fluxes

Condition	Time constant (min)	J_{Na} (fmoles/min)	n	p
HEPES buffer				
Efflux	0.48 ± 0.03	−12.40 ± 1.03	9	<0.05
Influx	0.66 ± 0.07	9.01 ± 1.08	9	<0.05
Bicarbonate buffer				
Efflux	0.44 ± 0.02	−14.38 ± 0.76	9	<0.05
Influx	0.28 ± 0.03	19.59 ± 2.16	9	<0.05
Hepes buffer + ouabain (5×10^{-4} M)				
Efflux	0.46 ± 0.02	−11.90 ± 0.74	13	NS
Influx	0.52 ± 0.04	11.44 ± 1.04	13	NS
Bicarbonate buffer + ouabain (5×10^{-4} M)				
Efflux	0.27 ± 0.02	−20.32 ± 1.27	10	NS
Influx	0.19 ± 0.02	28.87 ± 4.07	11	NS

The mean ± SEM is given for the Na fluxes calculated from the rate constant, k, according to Eq. 2. The number of monolayers is indicated by n. P value is for comparison of the absolute magnitudes of efflux and influx using the Student's t test.

olution differential interference contrast optical sections of the same cells and LIS were again obtained.

The area of each optical section was determined using a computer based edge detection algorithm (Marsh et al., 1985), and the volume of each cell and its adjacent LIS calculated. MDCK cell volume averaged 802.3 ± 125.6 fl (11 monolayers) in bicarbonate-buffered solution and was unaffected by ouabain over a 20 min experimental period. The ratio of cell volume in ouabain to the same cell in control solutions was 0.99 ± 0.01 (n = 11), not significantly different from 1.0. LIS volume was also not significantly altered by ouabain, with a ratio of experimental/control LIS volume = 1.08 ± 0.04 (n = 11) for the same LIS measured in ouabain and control solutions. This observation is in good agreement with previous reports that LIS geometry was unchanged by ouabain treatment (Chatton & Spring, 1995; Xia et al., 1995).

Effect of Ouabain on Na Transients in the LIS

The mathematical model predicts that the Na influx into the LIS should be significantly reduced by inhibition of the Na,K-ATPase. In a previous study (Chatton & Spring, 1995) perfusion of MDCK monolayers with solutions containing 5×10^{-4} M ouabain was used to reduce LIS Na concentration to values comparable to those in the bath.

After the control period in which images of SBFO fluorescence during Na efflux or influx were obtained with HEPES or bicarbonate-buffered solutions, the perfusate was switched to a solution of the same composition (142 mM Na) containing 5×10^{-4} M ouabain. After an equilibration time of at least 10 min, images were taken of the same cells at the same focal planes during the transients in LIS Na.

As shown in Fig. 4 and Table 2, Na influx into the

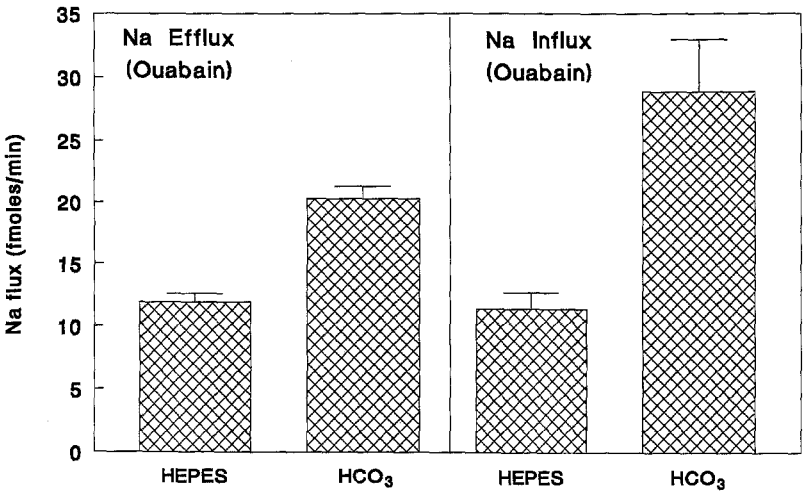

Fig. 4. Na fluxes into or out of the LIS in response to the perfusate Na changes in the presence of ouabain. Influx and efflux do not differ significantly from each other in HEPES or bicarbonate/CO_2. The number of experiments in HEPES was 13 for efflux and influx; in bicarbonate/CO_2, 10 experiments were done for efflux and 11 for influx determinations.

LIS in HEPES-buffered solutions in the presence of ouabain was equal to efflux, in good agreement with the model prediction. Both fluxes were not significantly different from those measured in the absence of ouabain. The fluxes were calculated from the measured rate constants and the previously determined LIS Na concentration (Chatton & Spring, 1995). These results are consistent with the previous conclusion that very little active Na transport occurs across MDCK cells in HEPES buffered solutions (Chatton & Spring, 1995).

The Na influx and efflux from the LIS in bicarbonate buffered solutions in the presence of ouabain were also not significantly different from one another (Fig. 4, Table 2). Na efflux was somewhat greater than under control conditions ($P < 0.01$), possibly reflecting an increase in tight junctional permeability secondary to changes in intracellular ionic composition. Na influx in the presence of ouabain did not differ significantly from that under control conditions (Table 2).

Regulation of TJ Permeability by cAMP Analogues

Because the regulation of tight junctional permeability by intracellular cAMP has been the subject of recent controversy (Kottra et al., 1993*a,b*) and it was possible to measure the Na flux through a localized region of tight junctions and adjacent LIS, we examined the effect of alterations in intracellular cAMP or protein kinase A activity on the permeability of the *TJ*. Control experiments gave similar Na flux rate constants for two or three successive measurements on the same LIS under control conditions. The strategy was to measure the Na permeability of the same tight junction before and after perfusion of the monolayers with Rp-cAMPS and Sp-cAMPS, analogues of cyclic AMP in which one of two exocyclic oxygen atoms in the cyclic phosphate moiety are replaced by sulfur. Rp-cAMPS is a competitive inhibitor of protein kinase A (type I and II). Sp-cAMPS is an activator of protein kinase A.

Inhibition of Protein Kinase A

After control measurements with bicarbonate-buffered solutions of high (142 mM) and low (24 mM) Na concentration, the monolayer was perfused with the solutions containing 0.1 mM Rp-cAMPS. Acquisition of images was performed every few minutes over 10 min at the same focal plane and place as control measurements. As shown in Table 3 and Fig. 5, Rp-cAMPS dramatically slowed Na efflux from the LIS compared to control. Na efflux, based on a time constant of 0.87 ± 0.04 min and a concentration gradient of 133 mM, was reduced by 43%. Na influx, calculated from the time constant of 0.67 ± 0.06 min and a concentration gradient of 118 mM, was similarly reduced by Rp-cAMPS.

Stimulation of Protein Kinase A

Perfusion of monolayers with 0.1 mM Sp-cAMPS for 10 min resulted in the rapid loss of all SBFO from the LIS. The time constant for dye loss was 2.27 ± 0.46 min (5 monolayers), sufficiently rapid to make it impossible to measure Na in LIS and consistent with the conclusion that the permeability of the tight junctions had been substantially increased. Control measurements without Sp-cAMPS showed a time constant for loss of the dye from the LIS of about 50 min. A lower concentration of Sp-cAMPS was then used to ascertain whether a balance could be found between increased junctional permeability to Na and rapid dye loss. Preliminary experiments showed that it was possible to measure Na changes in LIS when the Sp-cAMPS concentration in apical solu-

Table 3. Time constants and Na fluxes in the presence of Rp-cAMPS or Sp-cAMPS

Condition	Time constant (min)	J_{Na} (fmoles/min)	n	p
Bicarbonate buffer + Rp-cAMPS (0.1 mM)				
Efflux	0.87 ± 0.09	−7.11 ± 0.74	9	NS
Influx	0.67 ± 0.06	8.19 ± 0.77	9	NS
Bicarbonate buffer + Sp-cAMPS (0.01 mM)				
Efflux	0.32 ± 0.03	−19.32 ± 1.88	8	<0.01
Influx	0.21 ± 0.01	26.12 ± 1.22	9	<0.01

The mean ± SEM is given for the Na flux calculated from the rate constant, k, according to Eq. 2. The number of monolayers is indicated by n. P value is for comparison of the absolute magnitude of efflux and influx using the Student's t test.

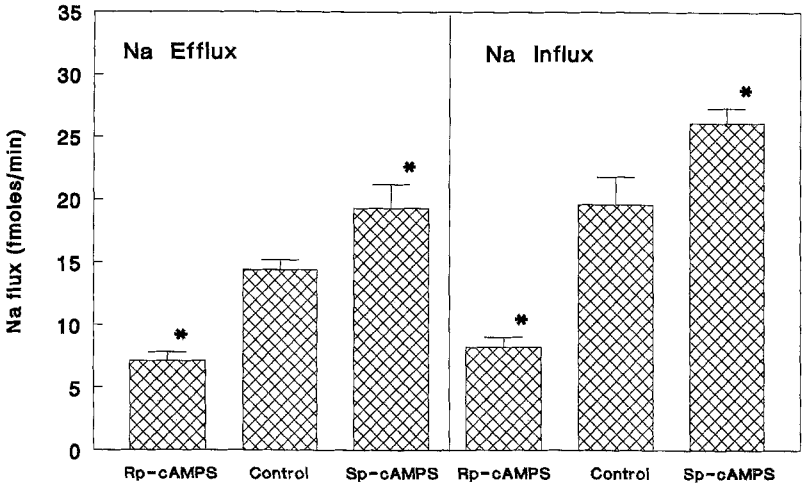

Fig. 5. Rp-cAMPS and SP-cAMPS effects on Na transients in the LIS in the presence of bicarbonate/CO_2 perfusates. Rp-cAMPS decreased both Na efflux and influx compared to control ($P < 0.001$). Na efflux and influx both increased significantly ($P < 0.02$) in the presence of Sp-cAMPS compared to control.

tion was 0.01 mM. In the presence of 0.01 mM Sp-cAMPS, the time constant for Na efflux from the LIS was reduced to 0.32 ± 0.03 min leading to a calculated Na efflux 34% higher than control (Table 3). Na influx was also increased 33% by Sp-cAMPS based on the time constant of 0.21 ± 0.01 min (Fig. 5 and Table 3).

Discussion

This study was motivated by the previous observation from our laboratory (Chatton & Spring, 1995) that transients in LIS Na were far slower than those for Cl (Xia et al., 1995). Two concerns were raised — that our MDCK cells differed in an unknown way from the cation-selective monolayers studied by others, or that our choice of NMDG as a Na substitute was inappropriate. The dilution and bi-ionic electrophysiologic experiments showed clearly that our MDCK cells had cation-selective tight junctions as reported by others (Oberleithner et al., 1990*a,b*). Further, it was clear that NMDG did not readily cross the tight junctions while another Na substitute, Li, did so as freely as Na. When the LIS Na transient experiments were repeated with Li as a Na replacement, the rate constants and calculated Na fluxes across the tight junctions increased more than tenfold. The results of these experiments yielded a number of insights about the properties of the tight junction and the LIS of MDCK cells.

Selectivity of MDCK Cell Tight Junctions

Our present results show that MDCK cell tight junctions are cation selective with a transference number for Na of about 0.84. The cation selectivity sequence of the tight junction may be deduced from a previous study on LIS pH (Harris et al., 1994) and the present optical and electrical experiments as Na = Li > NMDG > H. Although predominantly cation selective, the tight junctions allow passage of large anionic dyes (up to 1000 MW, R. Nitschke and K.R. Spring, *unpublished*). In previous studies, the LIS could not be loaded with cationic dyes regardless of their size (Harris et al., 1994) and tight junctional permeability to H was extremely low (Chatton & Spring, 1994). Thus, the picture which emerges is of a tight junctional permeability barrier with many, somewhat selective cation channels and fewer, nonselective anion channels.

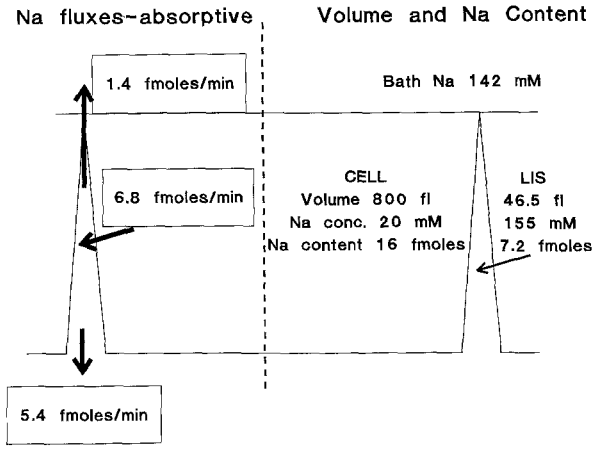

Fig. 6. Calculated Na fluxes and compartment sizes under control conditions based on the experimental measurements of cell and LIS volume, estimated Na concentration in the cell, and measured Na concentrations in the LIS and the bath.

Na Flux Estimation

The transcellular and transjunctional Na fluxes can be estimated by application of the mathematical model in Fig. 1 to the Na fluxes derived from the LIS Na transients. The results of these calculations are shown in Fig. 6. MDCK cell volume was measured in the present study as 802 fl, and if cell Na is about 20 mM/l, cell Na content would be ~16 fmoles. LIS volume was 46.5 fl, calculated as 5.8% of cell volume; measured LIS Na concentration was 155 mM/l yielding a Na content for the LIS of 7.2 fmoles. LIS Na content is about 45% of that estimated for the cell. The actively transported Na flux from cell-to-LIS in bicarbonate-buffered solutions is 6.8 fmoles/min, about 43% of the total cell Na content per minute. Approximately 1.4 fmoles/min of the transported Na leaks back to the apical bath across the tight junction. This back flux constitutes about 20% of the active Na efflux from cell-to-LIS, leading to a net Na efflux from the LIS of 5.4 fmoles/min. The flux estimates are in remarkably good agreement with those of Xia et al. (1995) for Cl in which the back flux of Cl from LIS to apical bath was calculated as 0.7 fmoles/min and the Cl flux from cell-to-LIS as 6.2 fmoles/min. The estimated net Cl flux out of the LIS was 5.5 fmoles/min, virtually identical to that calculated for Na in the present study (Fig. 6).

LIS and Cell Volume in the Presence of Ouabain

Ouabain inhibition of the Na,K-ATPase failed to change either MDCK cell or LIS volume significantly. Swelling of epithelial cells is a common occurrence after Na pump inhibition by ouabain, and many previous studies have utilized this cell swelling to delineate the mechanisms of NaCl entry across the apical membrane. Cell swelling results from the increase in intracellular NaCl after Na exit is inhibited (Spring & Hoffmann, 1992). The lack of effect of ouabain on MDCK cell volume could indicate that the rate of Na entry and exit are virtually zero or that the loss of cell K just balances the rate of Na entry. Because LIS Na concentration was elevated above that of the bathing solution only in bicarbonate buffer, the present experiments employing ouabain were done in the presence of bicarbonate to maximize the likelihood of detecting an effect of ouabain on cell or LIS volume. We speculate that MDCK cell volume is stable for ~20 min in the presence of ouabain because of a balance between K loss and Na gain rather than a complete lack of Na entry into the cells.

The stability of LIS volume and geometry in the presence of ouabain has been noted before (Chatton & Spring, 1995; Xia et al., 1995) and was confirmed in the present study. Since a previous investigation reported a highly deformable LIS in the *Necturus* gallbladder (Spring & Hope, 1978), the stability of the MDCK LIS in the presence of ouabain may seem somewhat surprising. However, rigidity of the lateral cell membranes and mechanical stability of the LIS in MDCK cells were also observed in a study of the water permeability and mechanical properties of MDCK cell membranes and LIS (M. Timbs and K.R. Spring, *in preparation*).

Ouabain inhibition of the transcellular component of Na influx into the LIS was used as a tool to estimate the magnitude of that component. Although influx and efflux were not statistically different in the presence of HCO_3, both fluxes were elevated compared to those under control conditions (Table 2). Such a result is consistent with an increase in tight junctional Na permeability associated with Na,K-ATPase inhibition. The mechanism of this increase was not investigated, but the experiments involving PKA stimulation and inhibition show clearly that *TJ* permeability is subject to regulation.

Regulation of Tight Junctional Permeability

The possibility of regulation of the permeability of epithelial tight junctions is in dispute. In an early study of *Necturus* gallbladder (Duffey et al., 1981) reported that tight junctional permeability, as assessed by transepithelial electrical resistance, was slowly increased by addition of cAMP to the bathing solutions. Recent papers by Kottra et al. (1993*a,b*) disputed the earlier findings in *Necturus* gallbladder and attributed the increase in transepithelial resistance to collapse of the LIS induced by cAMP. Other studies of intestinal epithelia (Bakker & Groot, 1984, 1989; Holman et al., 1979) and rabbit proximal tubule (Jacobson, 1979; Lorentz, 1974) reported that cAMP reduced transepithelial resistance. Of concern in all of these experiments was the possibility that

the observed resistance/permeability changes were the result of localized changes in cellular integrity or permeability rather than the uniform response of the tight junctions of the entire tissue.

Our experiments provide the first direct evaluation of the effects of both up- and downregulation of protein kinase A on tight junctional permeability. Since each experimental period was preceded by a control measurement of the same tight junction, concerns about effects on tissue integrity were abrogated. Our experiments clearly demonstrate a powerful effect of alterations of protein kinase A activity on the junctional tightness of MDCK cells. Stimulation of protein kinase A increased junctional permeability significantly while inhibition diminished permeability. The permeability increase induced by Sp-cAMPS seemed nonspecific, involving both Na and SBFO. An evaluation of the effects of Rp-cAMPS could only made on the Na permeability of the tight junctions as measurements of differences in the rate of dye loss were not feasible.

In summary, the permeability of the MDCK cell tight junction is about 5 times higher to Na than to Cl and is regulated by intracellular cAMP levels. Based on the changes in the rates of dye loss, it is likely that the cAMP regulation of junctional tightness is nonspecific. Our model calculations show that about 20% of the Na efflux from the cell during fluid absorption leaks back across the *TJ*, and that about 43% of the intracellular Na content is extruded every minute.

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