J. Membrane Biol. 144, 11–19 (1995)

The Journal of
Membrane
Biology

The Sodium Concentration of the Lateral Intercellular Spaces of MDCK Cells: A Microspectrofluorimetric Study

J.-Y. Chatton, K.R. Spring

Laboratory of Kidney and Electrolyte Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892-1598

Received: 14 July 1994/Revised: 19 October 1994

Abstract. MDCK cell monolayers grown on glass coverslips were used to examine the Na^+ concentration in individual lateral intercellular spaces (LIS) by video fluorescence microscopy. The LIS was filled with the Na^+-sensitive fluorescent dye SBFO by incubation of the monolayers for 75–90 min with 250 μM of the membrane impermeant form of the dye. After dye loading, the monolayers were perfused at 37°C with solutions buffered with HEPES or bicarbonate/CO_2 containing 142 mM Na^+. Ratios of the fluorescence images after sequential excitation with 340 nm and 380 nm light were performed and *in situ* calibration of LIS Na^+ was accomplished after blocking the Na^+ pump with 5×10^{-4} M ouabain. Measurements of Na^+ along the basolateral-to-apical axis of the LIS at 1.0 or 1.5 μm intervals did not reveal a Na^+ gradient when the perfusate was either HEPES or bicarbonate/CO_2 solutions. In bicarbonate solutions, the mean Na^+ concentration (mM) was 157.2 ± 2.3, ~15 mM higher than the bath Na^+ concentration. In HEPES solutions, however, the Na^+ concentration was not different from the bath concentration (142.7 ± 3.1 mM). The time course of Na^+ changes in LIS was investigated by rapidly switching the perfusate from 142 to 80 mM Na^+ and measuring the Na^+ changes at one focal plane.

Key words: Epithelia — Fluid transport — Fluorescence microscopy — Tissue culture — Na^+ transport

Introduction

Active transport of Na^+ by the Na^+,K^+-ATPase of epithelia is the primary driving force for the solute accumulation which drives transepithelial fluid transport (Schafer, 1990; Spring, 1991). Na^+ which enters the epithelial cell through the apical membrane is subsequently extruded across the basolateral cell membrane by the ouabain-sensitive Na^+,K^+-ATPase (Weinstein, 1992). An elevated NaCl concentration in the lateral intercellular spaces (LIS) of absorptive epithelia is an essential constituent of the increased osmotic pressure within the LIS proposed in the prevailing model for fluid transport — the standing gradient hypothesis (Diamond & Bossert, 1967). The magnitude of the osmotic gradient required for fluid absorption has proven difficult to determine experimentally and has been the subject of considerable controversy and speculation (Schafer, 1990; Weinstein, 1992). Direct determination of the Na^+ concentration within the LIS of fluid transporting epithelia has been limited to two studies. Simon et al. (1981) measured LIS Na^+ in *Necturus* gallbladder by the use of ion-sensitive microelectrodes. They found LIS Na^+ to be 6% greater than that of the bathing media, but expressed serious reservations about the precision of their measurements because of concerns about tip potentials and mechanical disturbances. Zeuthen (1983) used a similar approach to estimate LIS Na^+ in *Necturus* gallbladder bathed in very low osmolality solutions. He concluded that LIS Na^+ was indistinguishable from that of the bathing solutions.

Previous experience (Chatton & Spring, 1994; Harris et al., 1994) with the use of the fluorescent indicator for pH, BCECF, in cultured renal epithelial cells led us to attempt to measure LIS Na^+ in MDCK cells. Preliminary experiments in the spectrofluorimeter and fluorescence microscope showed that SBFI, the fluorescent probe generally used for intracellular Na^+ determinations (Harootunian et al., 1989), was not a suitable indicator for the Na^+ concentrations expected in the LIS. A related dye, SBFO (Minta & Tsien, 1989), proved to be usable in

Correspondence to: K.R. Spring

the high Na^+ concentration range of the extracellular fluid of mammalian epithelia. Although previous studies of the pH of the LIS could be performed on MDCK cells grown either on glass coverslips or on permeable supports, the present studies were limited to cells grown on glass coverslips. LIS Na^+ was significantly greater than that of the bathing medium only when HCO_3^- was present.

Materials and Methods

Cell Culture

Low resistance MDCK cells, passage 63–75 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 7–14 days.

Experimental Solutions and Perfusion System

The 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 7.4 at 37°C (Gueffroy, 1990); they 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 7% CO_2/93% air. The 80 mM Na^+ solution contained 62 mM NMDG-Cl (N-methyl-D-glucamine chloride, Aldrich, Milwaukee, WI) as a NaCl replacement. The osmolarity of all solutions was 292–300 mOsm/kg. The cell monolayers were perfused in a closed chamber (Harris et al., 1994), designed for rapid solution exchange. 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.

Materials

SBFO (ammonium salt) was obtained from Molecular Probes (Eugene, OR). Ouabain octahydrate was purchased from Sigma (St. Louis, MO) and weighed just before the experiment. Bovine albumin (fraction V) was from Miles (Kankakee, IL).

Choice and Spectral Characterization of the Na^+ Probe

The Na^+-sensitive fluorescent dye SBFO (sodium-binding benzofuran oxazole), first described by Minta and Tsien (1989) was selected for its high dissociation constant for sodium (K_D ~50–95 mM). The spectral properties and Na^+ sensitivity of SBFO were investigated on a fluorescence spectrometer (FluoroMax, Spex Industries, Edison, NJ) equipped with a miniature magnetic stirrer. For these measurements, the dye was dissolved in a solution buffered to pH 7.3–7.4 with 14 mM HEPES and NaCl was added to the 2 ml cuvette from concentrated solutions (1 or 4 M). Spectra were corrected for dye dilution following the additions of concentrated NaCl.

Microspectrofluorimetry

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 50 W tungsten halogen lamp (Leica).

The cell monolayers were observed through a 100×/1.3 N.A. objective lens (Nikon, Melville, NY), a microchannel plate intensifier (KS-1381, Videoscope, Washington, D.C.) and video camera (VS-2000N, Videoscope). An 8-frame running average was used to reduce the noise level of the image (LKH 9000, Videoscope) which was stored on an optical memory disc recorder (OMDR, TQ-2028F, Panasonic, Newark, NJ) for later offline analysis. Optical sections, each consisting of a 340 nm, a 380 nm and a bright field image, were recorded at 1 or 1.5 μm focal plane displacements. The focus was moved using a computer-controlled stepper motor, allowing focus displacement with 0.25 μm precision at a speed of 9–10 msec/μm.

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

To incorporate fluorescent dyes into the LIS, we used a modification of the strategy described previously for measuring the pH of LIS using the fluorescent probe BCECF (Chatton & Spring, 1994; Harris et al., 1994). Briefly, the cell monolayers were incubated in their culture dish for 75–90 min with the free acid SBFO (~250 μM in buffered experimental solutions with 140 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. It is worthy of note that loading the LIS with SBFO was much slower than BCECF (typically a 1–2 min loading time), probably because of the larger size of the molecule (MW 707 g/mol *vs.* 520 g/mol for BCECF); this shows the limitation of this method to relatively small fluorescent probes.

Image Analysis

Images were transferred from the optical disc recorder to an image analysis workstation (Trapix, 55/48Q, Recognition Concepts, Incline Village, NV) and the video images were converted into 8-bit digital images. Because a substantial heterogeneity of intensity ratios was found in the different segments of LIS around a given cell, analysis of the images was performed in two steps. First, a false-color ratio image of the LIS was produced from the 340 nm and 380 nm images after thresholding of the original images. Typically, four ratio images were produced along the time course of an experiment and used to select a region of LIS that presented a uniform ratio over its entire area. This selected area of LIS was then analyzed as described previously (Harris et al., 1994). Briefly, segmentation of the bright LIS and dark cellular

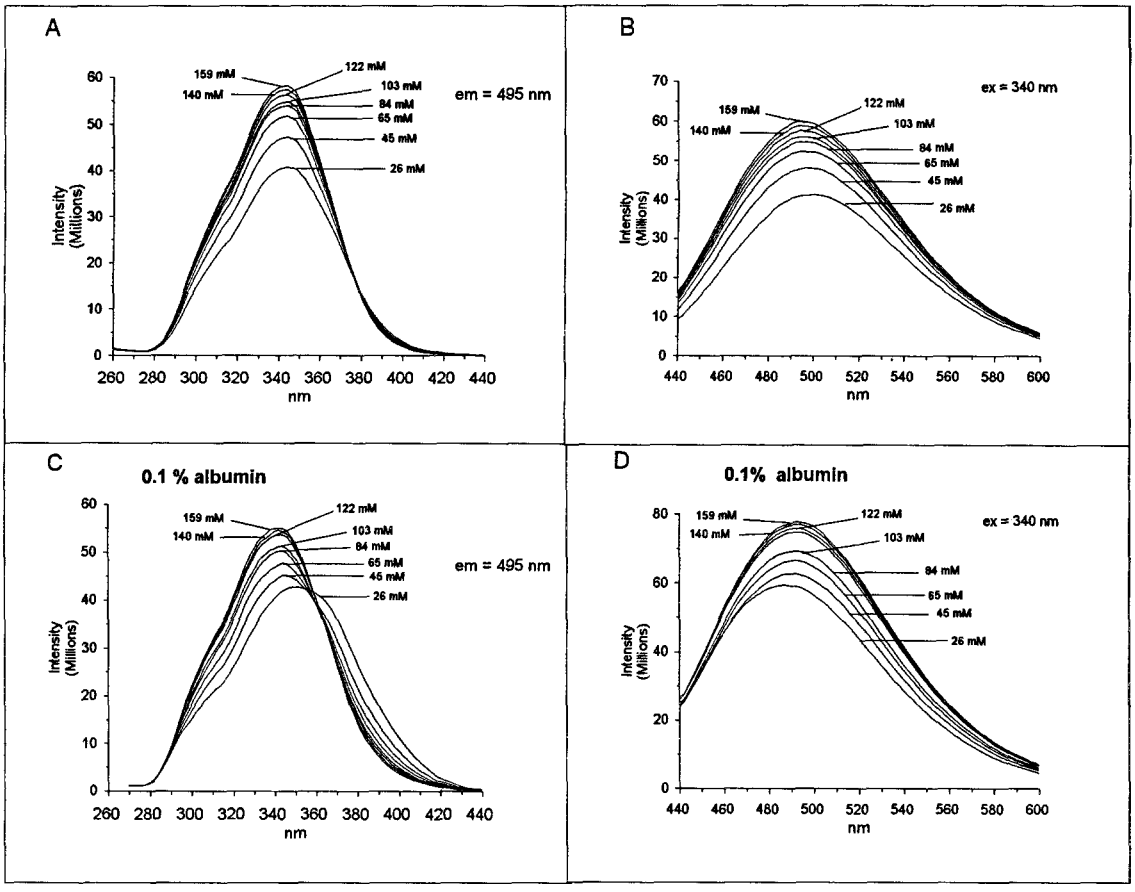

Fig. 1. Spectroscopic properties of SBFO. (*A*) Excitation spectra of SBFO with different Na^+ concentrations (26–159 mM). The emission monochromator was set to 495 nm. (*B*) Emission spectra corresponding to *A*. The excitation monochromator was set to 340 nm. (*C*) Excitation spectra in the presence of different Na^+ concentrations and 0.1 g% albumin. (*D*) Emission spectra corresponding to *B*.

regions was obtained from a binary image after thresholding of the 380 nm image. Regions of the LIS separated by small intensity differences 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.

Statistics

Data are presented as means ± SE. Statistical significance was determined using the Student's *t* test and a P value < 0.05 was considered significant.

Results

Spectral Characteristics of SBFO

SBFO (sodium-binding benzofuran oxazole) was first described by Minta and Tsien (1989) as a Na^+-sensitive fluorescent dye. Because of its high dissociation constant for sodium (K_D ~50–95 mM) and because its acetoxymethyl ester was found not to load cells properly, this dye was considered inappropriate for intracellular sodium determinations, for which a close relative, SBFI (sodium-binding benzofuran isophthalate), is now being used. For our studies of extracellular Na^+, SBFO appeared more promising than SBFI, because the levels of Na^+ in the LIS were expected to be relatively close to plasma levels (>100 mM) and because the quantum yield of SBFO was about five times higher than SBFI (Minta & Tsien, 1989).

Na^+ dependency of SBFO excitation spectrum is depicted in Fig. 1*A*. These spectra with Na^+ ranging from 26 to 159 mM show an intensity increase of the fluorescence maxima (~340 nm) and an isosbestic point at ~376 nm. The corresponding emission spectra (Fig. 1*B*) had an intensity maximum at ~495 nm and did not present any isosbestic point.

Several studies reported that SBFI behaved differently in the protein-rich intracellular (Harootunian et al., 1989; Borin & Siffert, 1991; Borin, Goldman & Blaustein, 1993) or intramitochondrial environments (Jung, Apel & Brierley, 1992) than in a cuvette. Because our previous study (Chatton & Spring, 1994) gave good indications of the presence of pH-stabilizing factors, pre-

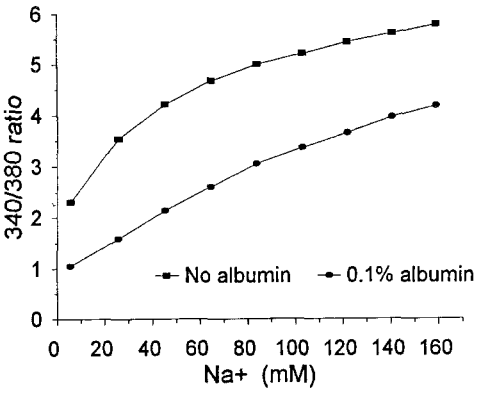

Fig. 2. Fluorescence excitation ratios of SBFO (340/380 nm) calculated from Fig. 1 in the presence (-●-) or the absence (-■-) of 0.1 g% albumin. Emission was detected at 495 nm.

sumably glycoproteins, in the LIS of MDCK cells, SBFO was tested for its sensitivity to proteins. Albumin was used to simulate this environment. Figure 1*C* shows a Na^+ calibration in the presence of 0.1% (w/v) albumin. Although the wavelength of the excitation maximum remained unchanged, the isosbestic point for Na^+ was shifted from ~376 to ~360 nm. Also, the excitation curves at wavelengths longer than the isosbestic point, close together in the absence of sodium (Fig. 1*A*), became clearly separated in the presence of albumin. The emission spectrum was not significantly altered by the presence of albumin (Fig. 1*D*).

The physico-chemical origin of the protein effect remains uncertain, but these measurements point to an inherent problem in the use of this fluorescent dye: without knowing the exact concentration of proteins in a preparation, the signal calibration for Na^+ has to be done *in situ,* provided that the protein concentration stays constant during the measurements and the calibration procedure.

SBFO was then tested for potential interference by other factors in the concentration range expected for the extracellular fluid, namely K^+ (0–20 mM), Ca^{2+} (0–10 mM), Mg^{2+} (0–5 mM) and pH (6–8). SBFO was dissolved in the same buffer as described above but containing 100 mM Na^+. These measurements revealed that SBFO was insensitive to all these factors. In addition, SBFO also proved to be insensitive to ouabain at the concentration used in the experiments (5×10^{-4} M), and presented the same response for Na^+ when the solutions were buffered by bicarbonate/CO_2 instead of HEPES.

To examine whether the effect of increasing Na^+ on SBFO fluorescence was independent of ionic strength, solutions were prepared that contained NMDG to provide a constant cation concentration ([Na^+] + [NMDG] = 160 mM). In such solutions, SBFO gave the same result as in solutions not corrected for ionic strength, both in the absence and in the presence of 0.1% albumin.

Figure 2 shows the fluorescence excitation ratio of signals 340/380 nm as a function of Na^+ concentration calculated from the curves of Fig. 1. It indicates that, in the absence of proteins, the ratio increased with a steep slope up to about 60 mM Na^+ and tended to flatten out at higher concentrations. In the presence of proteins, the 340/380 nm ratio was more linear and presented a steeper increase at higher Na^+ concentrations. This favorable effect was due to the more pronounced spectral shift induced by Na^+ in the presence of proteins.

Imaging Na^+ in the LIS

SBFO was loaded in the LIS using a modification of the technique used previously for the fluorescent pH probe BCECF (Chatton & Spring, 1994; Harris et al., 1994). It was found that BCECF could be loaded in the LIS by briefly perfusing the apical side of the epithelium. Passive diffusion of the fluorescent dye across the tight junctions would gradually fill the LIS (as well as the domes), providing a clearly defined fluorescent signal originating from the LIS while the neighboring cells would remain dark. In the BCECF experiments, the loading time was short (typically 1–2 min) and performing in the perfusion chamber. As a comparison, loading SBFO in the LIS of MDCK cells proved more difficult, probably because of the different size and shape of the two molecules. A much longer time was found to be required to successfully load SBFO (>30 min); therefore, instead of performing the loading step in the perfusion chamber, the cells were treated directly in the culture dish containing the coverslip by adding SBFO to the culture medium to a final concentration of ~250 μM. The incubation, typically 75–90 min, was done in the incubator at 37°C and resulted in satisfactory images of the SBFO-loaded LIS (Fig. 3, upper panels). A comparison of the fluorescence images with the bright field image of the cells (Fig. 3, lower left panel) demonstrated the restriction of the fluorescent signal to the LIS.

Fluorescence excitation ratios (340/380 nm) obtained from LIS or from domes of MDCK cells were found to be substantially different. Significant differences in ratio could also be found around a single cell, often related to wider areas of the LIS. We postulate that this phenomenon was not due to differences in Na^+ concentration but to the effect of proteins on SBFO described in the preceding section. Therefore, the image analysis strategy in our previous investigations with BCECF (Chatton & Spring, 1994; Harris et al., 1994), in which the average fluorescence ratio of the LIS around an entire cell was used, could not be readily applied. Instead, in a first step, a fluorescence ratio image was created using the threshold images obtained at 340 nm and 380 nm. Critical observation of 3–4 color-coded ratio images (Fig. 3, lower right panel) of the same cell taken at the end of each experimental period allowed us to select regions of LIS that did not show detectable

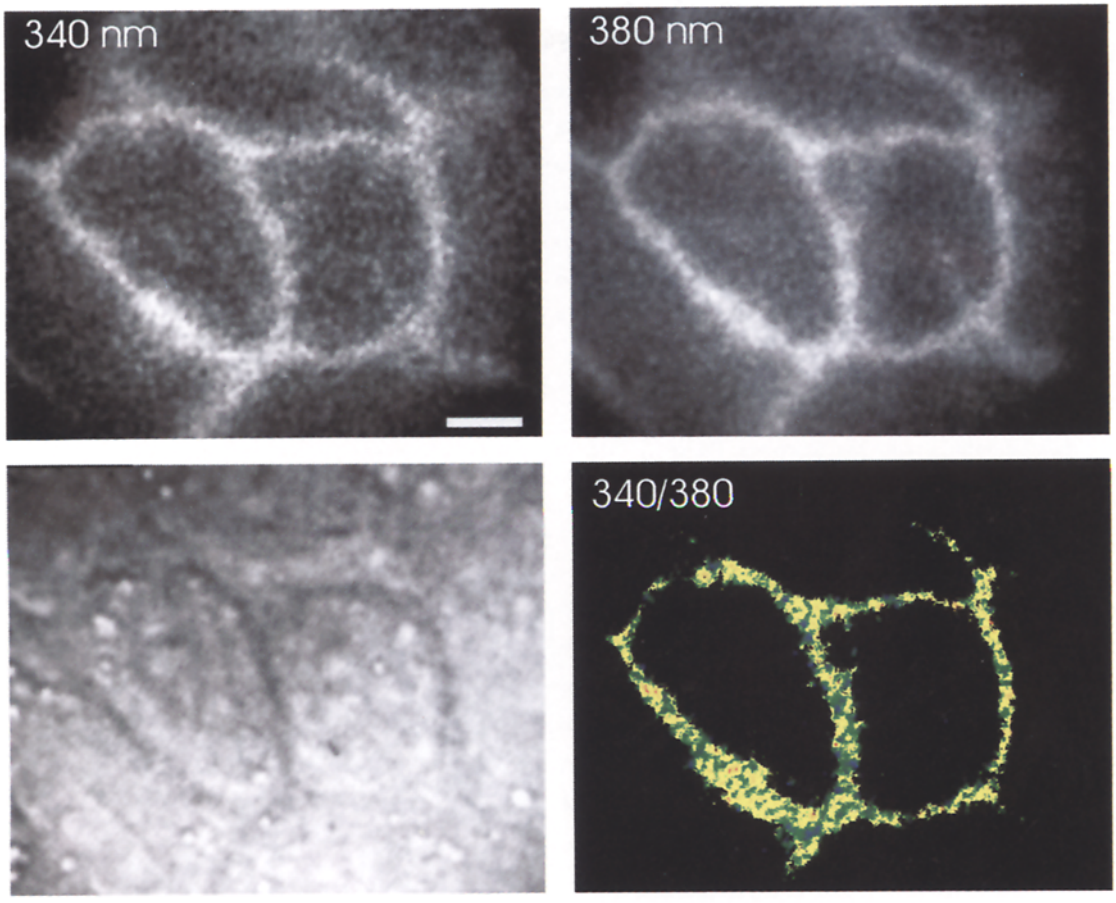

Fig. 3. LIS of MDCK cells loaded with SBFO. (Upper panels) fluorescence images (8 frame average) obtained at 340 nm and 380 nm. (Lower left panel) bright field image of the MDCK cells, showing the colocalization of the fluorescent signal with the interspaces. (Lower right panel) pseudocolor image obtained by ratioing the 340 nm and the 380 nm images.

geometry changes and had uniform ratios. These selected regions were then used for subsequent Na^+ concentration determinations.

LIS Na^+ in HEPES and Bicarbonate/CO_2 Solutions

In a first series of experiments, MDCK cells were studied in HEPES-buffered solutions. After the cells were mounted in the chamber, they were perfused for 15–30 min before the acquisition of images was started to wash the dye out of the apical bath and allow equilibration of the tissue in the perfusion solution. Images (transmitted, 340 nm and 380 nm images) were captured for ~10 minutes. For each time point, three to four optical slices at 1.0 or 1.5 μm intervals were taken, starting at the base of the LIS.

As indicated above, because of the environmental sensitivity of SBFO, an *in situ* calibration had to be carried out for each experiment. The strategy was to block Na^+ transport by perfusion of the monolayers with ouabain and wait until LIS Na^+ reached a new steady state, with a Na^+ concentration now presumably equal to that of the bulk solution. Indeed, in the absence of Na^+ transport, Na^+ should reach equilibration with the bath, mostly by paracellular diffusion. Preliminary experiments showed, in agreement with previous studies (Simmons, 1981), that ouabain produced complete inhibition of the Na^+,K^+-ATPase of MDCK cells within 10 min. Therefore, the following calibration procedure was routinely done for each experiment (Fig. 4): after the control period, the perfusion solution 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 at selected focal planes. The procedure was then repeated with an 80 mM Na^+ solution with ouabain in which Na^+ was replaced by NMDG while Cl^- was unchanged. LIS Na^+ achieved a new steady state 10–15 min after switching the apical perfusate to the 80 mM Na^+ solution with ouabain. The ratio values obtained at these two calibration points at each focal plane, were used to calculate the Na^+ concentration in the control period for the corresponding optical section.

Using this approach, Na^+ concentration was measured over a 6 μm length of LIS, and, as Fig. 5 indicates,

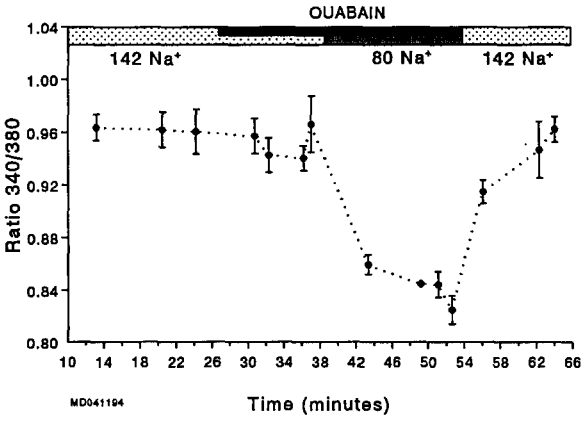

Fig. 4. Experimental record showing a calibration procedure. The ordinate is the mean 340/380 nm intensity ratio inside a selected region of LIS and the abscissa is time (minutes) started when perfusion was initiated. The monolayer was perfused in HEPES-buffered experimental solution with 142 mM Na^+. At 26.3 min the perfusate was switched to a solution of the same composition with 5×10^{-4} M ouabain added and at 38.4 min, the perfusate was switched to an 80 mM Na^+ solution with ouabain. Recovery was tested after switching the perfusion back to the initial solution. Data are means (±SE) of four optical sections along the LIS axis.

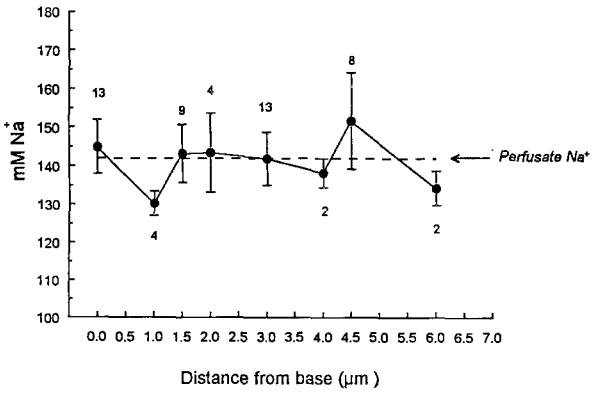

Fig. 5. Na^+ concentration in the LIS of MDCK cells perfused in HEPES solutions. Measurements of Na^+ were made along the LIS axis by optical sectioning. The dashed line indicates the Na^+ concentration of the perfusate. The number of LIS obtained from thirteen experiments is indicated in the graph.

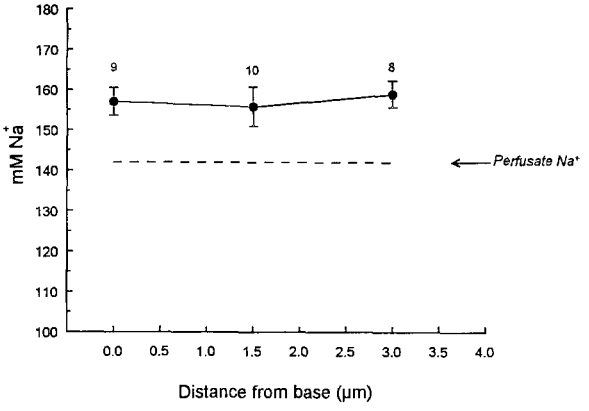

Fig. 6. Na^+ concentrations in the LIS of MDCK cells perfused in bicarbonate/CO_2 solutions. Measurements of Na^+ were made along the LIS axis by optical sectioning. The dashed line indicates the Na^+ concentration of the perfusate. The number of LIS obtained from ten experiments is indicated in the graph.

no sodium gradient was detected between the base of the cells and the tight junctions. The average Na^+ concentration found in the LIS in the HEPES-buffered solutions was 142.7 ± 3.1 mM (57 optical sections from 13 monolayers), not different from the perfusate solution.

The same approach and calibration procedures were undertaken with MDCK cells perfused with bicarbonate/CO_2 solutions. Figure 6 shows that, as in HEPES solutions, no Na^+ gradient was detected along the region of the basolateral-to-apical axis of the LIS from which accurate Na^+ concentration measurements could be made. However, the LIS Na^+ concentration of MDCK cells perfused with bicarbonate/CO_2 solutions was 157.2 ± 2.3 mM (27 sections from 10 monolayers, $P < 0.001$), about 15 mM higher than in the HEPES-buffered solutions (Fig. 7).

Kinetics of Na^+ Changes

Experiments were then devised to evaluate the rate of Na^+ change in LIS when the perfusate Na^+ concentration was rapidly switched from a 142 mM Na^+ HEPES-buffered solution to a 80 mM Na^+ solution. Na^+ was replaced isosmotically by NMDG; the Cl concentration was unchanged. Acquisition of images was performed every 10–15 seconds at one focal plane only (at 2–3 μm from the base). Fig. 8*A* shows an example of changes obtained during such an experiment. The line drawn through the data points in Fig. 8*B* was obtained by non-linear curve fitting (Levenberg-Marquardt algorithm) using the following equation:

$$R = A + B \cdot e^{-\frac{t}{\tau}}$$

where R is the 340/380 nm ratio, t is the time, τ is the characteristic time, A and B are optimized parameters. In ten different monolayers, t averaged 4.8 ± 0.7 min. Switching the solution back to 142 mM Na^+ gave noisier data with τ values not significantly different from the ones obtained in the 142 – >80 mM Na^+ switch.

The result of these experiments implies that, in the absence of ouabain, 95% of the final steady state LIS concentration would be reached in 14.4 ± 2.1 min after switching the bath from 142 to 80 mM Na^+.

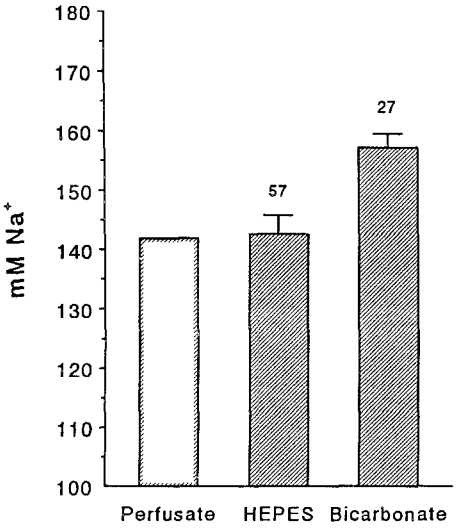

Fig. 7. Mean Na^+ concentrations in HEPES and bicarbonate/CO_2 solutions. Open bar is the perfusate Na^+ concentration. The number of LIS measured from thirteen (HEPES) and ten (bicarbonate) preparations is indicated above each bar.

Discussion

This study reports the first direct measurements of Na^+ concentration in the LIS of MDCK epithelial cells. Video fluorescence microscopy was chosen for its ability to permit noninvasive investigation of living specimens with high spatial and temporal resolution.

Validity of the Method

We took advantage of the methods that were developed for previous studies in our laboratory (Chatton & Spring, 1994; Harris et al., 1994) for the study of pH in the LIS of cultured renal cells. The difficulty of making such measurements originates mainly from the tiny size and configuration of the LIS compartment, opened on one end to the serosal compartment and closed on the other end by the tight junctions. Even when fluorescent probes were successfully loaded in the LIS of cells grown on nonpermeable supports, they gradually diffused out of the LIS through the tight junctions. Any maneuver that tends to loosen or compromise the integrity of the tight junctions will inevitably cause the dye to leak out. This problem makes it difficult to use the pore-forming antibiotics or ionophores generally used to equilibrate the intracellular milieu with the bathing medium. For this reason, the strategy selected to perform *in situ* calibration was to block the Na^+,K^+-ATPase using ouabain and allow the LIS Na^+ to equilibrate with the perfusate.

The accuracy of our measurements was limited mainly by the modest changes in ratios exhibited by SBFO when Na^+ varied in the concentration range 100–200 mM. This was a consequence of a K_D for Na^+ of only ~50–95 mM (Minta & Tsien, 1989). The protein effect was an equally important limiting factor as was previously reported for SBFI (Borin et al., 1993), where uneven ratios were found inside single cells due to differences in regional microenvironment, rather than Na^+ concentration. To circumvent this problem, a calibration scheme was used in which a region of LIS selected for its

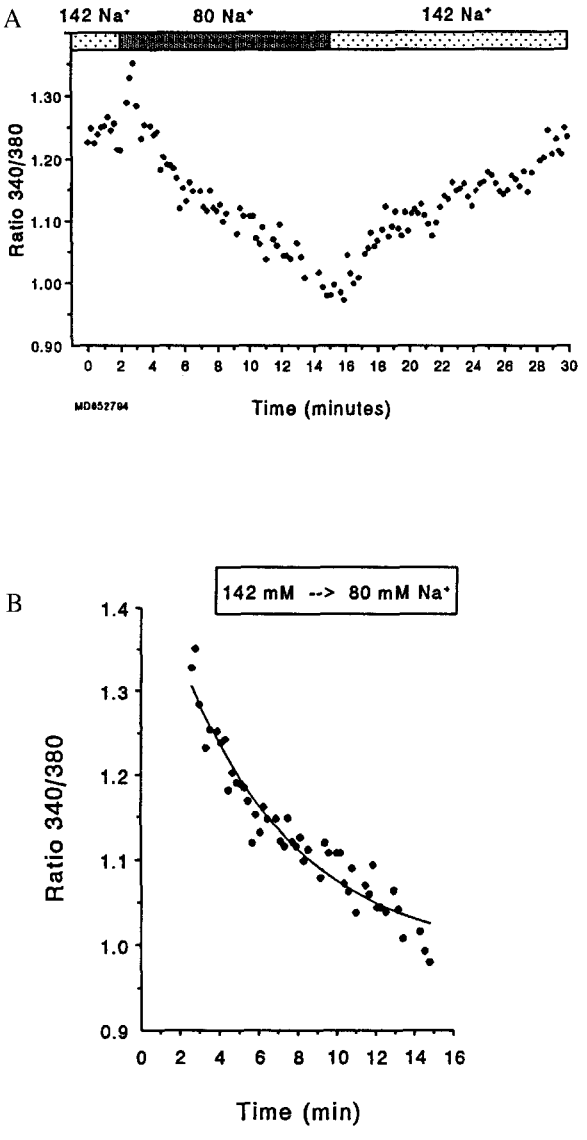

Fig. 8. Kinetics of Na^+ changes in the LIS following bulk perfusate Na^+ changes. (*A*) Experimental record showing 340/380 nm ratios measured at one focal plane while the perfusate was switched from 142 to 80 mM Na^+. (*B*) Nonlinear curve fitting from the 142 – >80 mM switch using the equation:

$$R = A + B \cdot e^{-\frac{t}{\tau}},$$

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

uniformity of fluorescence ratios was used throughout both the control period and the calibration steps, and each individual focal plane was treated separately.

A recent study reported estimates of interstitial Na^+ concentrations up to 650 mM in rabbit colonic crypts using the fluorescent Na^+ probe SBFI (Naftalin & Pedley, 1990; Pedley & Naftalin, 1993). We are concerned about the validity of these estimates on methodological grounds, because the measurements performed with a low magnification (16×) objective lens, assumed both that the K_D of SBFI for Na^+ (~7.4–18 mM (Minta & Tsien, 1989)) and the spectral characteristics of the dye were identical in the bulk solution and in the crypt microenvironment. In addition, a single wavelength of excitation was used, and no attempt was made to measure the local dye concentration or to calibrate the response of the dye to Na^+.

Na^+ Concentrations in the LIS

Our study showed that Na^+ concentration was higher (by about 15 mM, or ~10%) in the LIS than in the bicarbonate/CO_2 perfusion solution. This result is in agreement with studies done on *Necturus* gallbladder by Simon et al. (1981) using ion-sensitive microelectrodes in which the Na^+ concentration was found to be ~6% higher the LIS than in the perfusate, and Cl^- about 11% higher. Using the same approach, K^+ was also found to be higher in the LIS than in the bath (Curci & Frömter, 1979). Questions about the validity of these results were raised by the authors of these studies because of the extreme difficulty in puncturing the LIS without damaging the surrounding cells and the difficulty in avoiding leaky impalements. Indeed, using similar microelectrode techniques, Zeuthen (1983) was not able to find any concentration gradient of Na^+, K^+ and Cl^- between the bath and the LIS of *Necturus* gallbladder perfused in a low solution osmolarity (62 mOsm).

The present study was carried out on cells grown on coverslips, and it is conceivable that a gradient smaller than 15 mM between the LIS and the bath would be found if the cells had been cultured on a permeable support. Interestingly, in HEPES solutions, MDCK cells failed to maintain a higher Na^+ in the LIS. Thus, bicarbonate seems to play a critical role in the transepithelial Na^+ transport of MDCK cells. Cl^-/HCO_3^- transport has been reported in MDCK cells (Kurtz & Golchini, 1987; Oberleithner et al., 1990*b*) and could act in conjunction with Na^+/H^+ to produce such effects. Differences in the pH of the LIS of MDCK cells grown on glass coverslips in the presence and the absence of bicarbonate were also noted previously (Harris et al., 1994). It remains to be shown whether the higher LIS Na^+ in the presence of bicarbonate/CO_2 is accompanied by a higher fluid flow than in HEPES solutions. Transepithelial fluid flow in cells grown on impermeable supports is not directly measurable. The frequency of fluid-filled domes per monolayer, often used as an indicator of transepithelial fluid and solute transport (Oberleithner, Vogel and Kersting, 1990*a*; Kersting, Kersting and Spring, 1993), could not be used because the cells were kept in bicarbonate/CO_2-containing culture medium until the time of the experiment, and only then perfused with the experimental solution with or without bicarbonate.

Implications for the Standing Gradient Model

This study did not find a Na^+ gradient along the LIS of MDCK cells in either HEPES or bicarbonate/CO_2 solutions. This result confirmed a similar observation (Harris et al., 1994) for LIS pH, and may have implications for models of fluid transport. An important assumption of the Diamond and Bossert (1967) model was the presence of a gradient of solutes, mainly Na^+ and Cl^-, from the tight junctions to the base of the LIS. The magnitude of the standing gradient was proposed to be a function of the relative rates of active solute transport compared to diffusion. In this model, water drawn out of the cells by the osmotic gradient across the lateral cell membranes progressively dilutes the LIS contents, creating a standing gradient along the LIS. Diffusion of solutes and water tend to erase this gradient.

The present study tends to invalidate this hypothesis. One could argue that such a gradient would occur only with the cells on a permeable support. However, gradients of pH or BCECF were also not detected along the LIS of MDCK cells grown on filters (Chatton & Spring, 1994). Of critical concern in the present and previous studies is that the rate of fluid transport by MDCK cells is probably small compared to the gallbladder and renal proximal tubule. Thus any standing gradient could be obscured by diffusion.

Na^+ Transients

The time course of Na^+ changes after rapid switching of perfusate Na^+ revealed a remarkably slow process. In similar experiments on MDCK cells (Xia, Persson & Spring, 1995), the rate of Cl^- change in the LIS was almost one order of magnitude faster than that observed in the present study for Na^+. The routes taken by Na^+ could be both transjunctional and transcellular, however the transjunctional route would be expected to predominate. We had previously observed that MDCK cell tight junction permeability for protons was very low (Chatton & Spring, 1994; Harris et al., 1994), and we have been thus far unable to load the LIS with any catonic fluorescent dyes by diffusion across tight junctions. These observations are consistent with either very low junctional

cation permeability or a highly selective cationic pathway across the tight junction. Electrophysiologic studies by others (Oberleithner et al., 1990*a*, 1990*b* as well as in our laboratory, J.-Y. Chatton and K.R. Spring, *unpublished observations*) show that MDCK cell monolayers have cation-selective tight junctions which are poorly permeable to NMDG. Thus the slow time course of LIS Na^+ concentration change in our experiments is probably due to the low junctional permeability to NMDG. Additional experiments with other cation substitutes for Na^+ will be required to evaluate this hypothesis.

If accurate measurement of the rate of Na^+ change in the LIS were possible for both a 142 – >80 mM and a 80 – >142 mM Na^+ switch, this experimental protocol could enable the determination of the routes of Na^+ flux, i.e., the relative magnitudes of the transjunctional and transcellular fluxes. Indeed, the apparent rate of Na^+ decrease (efflux from the LIS) should be slower than the apparent Na^+ influx which is the additive result of transjunctional Na^+ flux and active transport by the Na^+,K^+-ATPase of the lateral membranes. Unfortunately, sufficient accuracy was not reached with our current techniques, because of Na^+ substitute used as well as the sensitivity of SBFO to variations in LIS geometry.

Future Directions

Clearly, measurement of Na^+ in LIS should be extended to epithelial cells cultured on permeable supports as well as to the renal proximal tubule or the gallbladder. These experiments will only be feasible if adequate techniques for loading fluorescent probes in the LIS of such preparations are developed.

In conclusion, the present study shows that MDCK cells were able to maintain a higher Na^+ concentration in the LIS than in the bath when perfused with a bicarbonate-containing solution, and that Na^+ concentration gradients were not present along the LIS axis.

We would like to thank P.H. Tran and C. Gibson for their technical and computational assistance as well as Dr. B.-E. Persson (University of Uppsala, Sweden) for his contribution in the early phases of the study.

References

Borin, M., Siffert, W. 1991. Further characterization of the mechanisms mediating the rise in cytosolic free Na^+ in thrombin-stimulated platelets. *J. Biol. Chem.* **266:**13153–13160

Borin, M.L., Goldman, W.F., Blaustein, M.P. 1993. Intracellular free Na^+ in resting and activated A7r5 vascular smooth muscle cells. *Am. J. Physiol.* **264:**C1513–C1524

Chatton, J.-Y., Spring, K.R. 1993. Light sources and wavelength selection for widefield fluorescence microscopy. *MSA Bull.* **23:**324–333

Chatton, J.-Y., Spring, K.R. 1994. Acidic pH of the lateral intercellular spaces of MDCK cells cultured on permeable supports. *J. Membrane Biol.* **140:**89–99

Curci, S., Frömter, E. 1979. Micropuncture of lateral intercellular spaces of Necturus gallbladder to determine space fluid K^+ concentration. *Nature* **278:**255–257

Diamond, J.M., Bossert, W.H. 1967. Standing-gradient osmotic flow. A mechanism for coupling water and solute transport in epithelia. *J. Gen. Physiol.* **50:**2061–2083

Gueffroy, D.E. 1990. Buffers: A Guide for the Preparation and Use of Buffers in Biological Systems. Calbiochem Corp., San Diego, California

Harootunian, A.T., Kao, J.P.Y., Eckert, B.K., Tsien, R.Y. 1989. Fluorescence ratio imaging of cytosolic free Na^+ in individual fibroblasts and lymphocytes. *J. Biol. Chem.* **264:**19458–19467

Harris, P.J., Chatton, J.-Y., Tran, P.H., Bungay, P.M., Spring, K.R. 1994. Optical microscopic determination of pH, solute distribution and diffusion coefficient in the lateral intercellular spaces of epithelia cell monolayers. *Am. J. Physiol.* **266:**C73–C80

Jung, D.W., Apel, L.M., Brierley, G.P. 1992. Transmembrane gradients of free Na^+ in isolated heart mitochondria estimated using a fluorescent probe. *Am. J. Physiol.* **262:**C1047–C1055

Kersting, U., Kersting, D., Spring, K.R. 1993. Ketoconazole activates Cl^- conductance and blocks Cl^- and fluid absorption by cultured cystic fibrosis (CFPAC-1) cells. *Proc. Natl. Acad. Sci. USA* **90:**4047–4051

Kurtz, I., Golchini, K. 1987. Na^+-independent Cl^--HCO_3^- exchange in Madin-Darby canine kidney cells. *J. Biol. Chem.* **262:**4516–4520

Minta, A., Tsien, R.Y. 1989. Fluorescent indicators for cytosolic sodium. *J. Biol. Chem.* **264:**19449–19457

Naftalin, R.J., Pedley, K.C. 1990. Video enhanced imaging of the fluorescent Na^+ probe SBFI indicates that colonic crypts absorb fluid by generating a hypertonic intersitial fluid. *FEBS Lett.* **260:**187–194

Oberleithner, H., Vogel, U., Kersting, U. 1990*a*. Madin-Darby canine kidney cells. I. Aldosterone-induced domes and their evaluation as a model system. *Pfluegers Arch.* **416:**526–532

Oberleithner, H., Vogel, U., Kersting, U., Steigner, W. 1990*b*. Madin-Darby canine kidney cells. II. Aldosterone stimulates Na^+/H^+ and Cl^-/HCO_3^- exchange. *Pfluegers Arch.* **416:**533–539

Pedley, K.C., Naftalin, R.J. 1993. Evidence from fluorescence microscopy and comparative studies that rat, ovine and bovine colonic crypts are absorptive. *J. Physiol.* **460:**525–547

Schafer, J.A. 1990. Transepithelial osmolality differences, hydraulic conductivities, and volume absorption in the proximal tubule. *Ann. Rev. Physiol.* **52:**709–726

Simmons, N.L. 1981. The action of ouabain upon chloride secretion in cultured MDCK epithelium. *Biochem. Biophys. Acta* **646:**243–250

Simon, M., Curci, S., Gebler, B., Frömter, E. 1981. Attempts to determine the ion concentrations in the lateral intercellular spaces between the cells of Necturus gallbladder epithelium with microelectrodes. *In:* Water Transport Across Epithelia: Barriers, Gradients and Mechanisms. H.H. Ussing, N. Bindslev, N.A. Lassen, O. Sten-Knudsen, editors. pp. 52–64. Munksgaard, Copenhagen

Spring, K.R. 1991. Mechanism of fluid transport by epithelia. *In:* Handbook of Physiology. The Gastrointestinal System, Volume IV. S.G. Schultz, editor. pp. 195–207. Oxford University Press, New York

Weinstein, A.M. 1992. Sodium and Chloride Transport: Proximal Nephron. *In:* The Kidney: Physiology and Pathophysiology. D.W. Seldin, G. Giebish, editors. pp. 1925–1973. Raven Press, New York

Xia, P., Persson, B.-E., Spring, K.R. 1995. The chloride concentration in the lateral intercellular spaces of MDCK cell monolayers. *J. Membrane Biol.* **144:**21–30

Zeuthen, T. 1983. Ion activities in the lateral intercellular spaces of gallbladder epithelium transporting at low external osmolarities. *J. Membrane Biol.* **76:**113–122