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Measurements of Intracellular pH in Single LLC-PK$_1$ Cells: Recovery from an Acid Load via Basolateral Na^+/H^+ Exchange

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Summary. LLC-PK$_1$ cells (a continuous epithelioid cell line with renal characteristics) are examined by microspectrofluorometry as single cells, in order to determine the mechanism of intracellular pH (pH_i) recovery from an acid load imposed by ammonium preincubation and removal (NH_4 prepulse). Initial experiments evaluate the intracellular K^+ levels through a null point analysis of total cellular K^+ with flame photometry. The response of BCECF (a pH-sensitive fluorescent dye) is then calibrated, using saturating concentrations of nigericin to cause defined changes in pH_i. For experiments with the microspectrofluorometer, LLC-PK$_1$ cells were grown on either glass coverslips or filters (the latter attached to plastic coverslips with a hole under the filter). The cells on glass coverslips demonstrate a Na^+-dependent recovery from an (NH_4 prepulse) acid load which is sensitive to 1 μM ethylisopropylamiloride. They also demonstrate a 'set point' of activation of Na^+/H^+ exchange. When examined for changes in pH_i due to changes in membrane potential, plasma membrane proton conductance could not be detected at resting pH_i. Cells grown on filters also demonstrate a pH_i recovery from an acid load which is Na^+ dependent and ethylisopropylamiloride sensitive, but in this configuration, the majority of cells (22/23 preparations) require Na^+ at the basolateral membrane for rapid pH_i recovery. The morphology and polarity of the cells grown on permeable supports appears normal at the electron-microscopic level. The results are not affected by changes in cell seeding density or collagen treatment of the filters.

Key Words: Na^+/H^+ exchange · intracellular pH · renal cell culture · LLC-PK$_1$ · membrane polarity · ethylisopropylamiloride

Introduction

Epithelial cells demonstrate a tissue-specific expression of membrane proteins. One form of cell specialization which is important to epithelial function is the expression of specific membrane proteins within a restricted domain of the plasma membrane (i.e. the apical or basolateral membrane). The functional asymmetry of epithelial cells is the basis for the performance of net (transcellular) transepithelial transport. In cases such as Na^+/glucose cotransport, the form of specialization is clear. Except for the brush-border membranes of the proximal tubule and small intestine, this transport activity is not detectable in the plasma membrane of mammalian tissue cells (Murer & Kinne, 1980). In other cases, the mechanism of specialization is less clear, because the transport system is present in both polarized and nonpolarized cells, but has an adapted use in epithelial cells (e.g. Na^+/H^+ exchange, Na^+-K^+-$2Cl^-$ cotransport) (Saier & Boyden, 1984; Mahnensmith & Aronson, 1985).

In both epithelial and nonepithelial cell types, Na^+/H^+ exchange is an important membrane transport system for cell pH homeostasis in the defense of intracellular pH (pH_i) against metabolic acid production (Roos & Boron, 1981). In some mammalian epithelia (e.g. the renal proximal tubule, gallbladder, and small intestine), evidence suggests that Na^+/H^+ exchange which is present in the brush-border membrane is directly involved in transepithelial Na^+ absorption and proton secretion (Murer et al., 1976; Aronson, 1981; Weinman & Reuss, 1984; Howlin et al., 1985; Knickelbein et al., 1985), and indirectly in either bicarbonate or Cl^- absorption (Murer & Burckhardt, 1983; Reuss & Costantin, 1984; Knickelbein et al., 1985).

Very little is known about the role of Na^+/H^+ exchange in pH homeostasis in epithelial cells, although it has been demonstrated that intracellular protons have a kinetic effect on the activity of the Na^+/H^+ exchange reaction in both epithelial and nonepithelial cells (Aronson et al., 1982; Grinstein & Rothstein, 1986; Montrose & Murer, 1986; Nord et al., 1986). Until recently, information on the localization and activity of Na^+/H^+ exchange was based on measurements in intact tissue or isolated membranes (Murer et al., 1976; Aronson et al., 1982; Ives et al., 1983; Sabolic & Burckhardt, 1983; Mircheff et al., 1984; Weinman & Reuss 1984; Howlin et al., 1985; Knickelbein et al., 1985). It is equally important to evaluate the function of the Na^+/H^+ exchange as it directly effects intracellular

pH. With the advent of pH microelectrodes and fluorescent dyes which monitor intracellular pH, it has become possible to follow intracellular pH more directly and at the single cell level (Rink et al., 1982; Alpern, 1985; Chaillet & Boron, 1985; Chaillet et al., 1985; Sasaki et al., 1985).

We have applied a microspectrofluorometry system to examine the polarized distribution of a transport system which controls pH_i in epithelioid tissue culture cells. The current work examines pH_i regulation in single LLC-PK_1 cells (epithelioid cells from pig kidney; Hull et al., 1976) grown on either glass coverslips or permeable supports. Cells grown on permeable supports are used with a separate perfusion of both the apical and basolateral aspects of the cells. In these cells, an examination of the requirements for pH_i recovery from an intracellular acid load defines the polarized distribution of Na^+/H^+ exchange with respect to its contribution to pH homeostasis in the LLC-PK_1 cell.

Abbreviations

BCECF, 2′,7′-*bis*-(2-carboxyethyl)-5,6-carboxyfluorescein; HEPES, 4-(-2-hydroxyethyl)-1-piperazine ethansulfonate; pH_i, intracellular pH; TMA^+, tetramethylammonium; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; EIPA, 5-(N-ethyl-N-isopropyl-amiloride; filterslip, a construction of a filter attached to a plastic coverslip; K_i^+, intracellular K^+ concentration; E_m, membrane potential.

Materials and Methods

Cell Culture

LLC-PK_1 cells (serial passage 148) were originally purchased from Flow Laboratories (Irvine, Scotland) and experiments were performed using cells between passage 156 and 166. Cells were maintained in culture and subcultured as described previously (Montrose & Murer, 1986). For experiments using microscopy, cells were seeded onto either sterilized glass coverslips or filterslips (filters attached to plastic coverslips, *see below*). After seeding, cells were left at 37°C for 1.5 to 3 hr and then unattached cells were removed and the coverslips returned to culture. Cells on filterslips had access to culture medium from both sides of the filter. When using glass coverslips, cells were seeded at a density (3×10^4 cells/coverslip) which allowed formation of a confluent monolayer in the third day after seeding. When filterslips were used, cells were seeded either at confluent density (2×10^5 cells/filterslip) and used 1 to 3 days after seeding or were seeded at 30-fold lower density (confluency in 4 to 6 days) and used 5 to 7 days after seeding. For experiments using flame photometry, 1.3×10^5 cells in 2 ml of culture medium were seeded onto 35-mm plastic dishes (Nunc, Denmark) and grown for two days prior to use.

Preparation of Filterslips

Plastic coverslips (22 × 22 mm, Fisher Scientific) with a 1.5-mm hole punched in the center were used for attachment of polycarbonate filters (0.8 or 1.0 μm pore size, Nucleopore). For attachment, filters were placed over the hole and a second coverslip was dipped in acetone and then drawn over the filter (similar to a blood smear). The resulting construction is referred to as a filterslip. Filterslips were sterilized by >2 hr in 70% ethanol and then left to dry overnight. They were then either used directly for cell seeding or were collagen treated. For collagen treatment, 40 μg/ml of rat tail collagen in 0.002% acetic acid (Serva) was placed on the filterslip for 30 min at 37°C and the filterslips were washed once in sterile distilled water and left to dry overnight. This procedure should result in nonspecific absorption of collagen fibrils to the plastic surface (Oebrink, 1982). We could not produce a homogeneous coating of renatured collagen fibers in a thickness which did not interfere with both the fluorescent measurement (Robins, 1983) and the permeability of the filter (*data not shown*).

Chamber and Perfusion

Cells either on glass coverslips or filterslips were mounted into the stainless steel chamber which is diagrammed (not to scale) in Fig. 1. The various components were held together by gently heating the chamber, applying paraffin wax of low melting point (50 to 52°C occluding point, Fluka) and then letting the chamber cool. During mounting of cells in the chamber, the solution bathing the cells does not rise above 39°C (*data not shown*). In Fig. 1, the chamber is shown for use with filterslips and independent perfusion of the 'apical' (~165 μl) and 'basolateral' (~300 μl) compartments. In this configuration, cells are attached only on the side of the plastic coverslip which faces the bottom glass coverslip and the objective of the inverted microscope. This results in a perfect epifluorescent image, but a poor transmitted light image (under optimal conditions, only cell outlines are visible). If a glass coverslip with cells is used, it is mounted into the 'apical' compartment using two other glass coverslips to contain it (the filterslip in Fig. 1 is replaced with a standard glass coverslip). In this configuration, the compartment has a volume of ~110 μl.

Stocks of perfusion solutions were kept in polyethylene bags (Semandeni, Switzerland) which were plugged with Lucite® corks allowing entry of Teflon® tubing (1.0 mm inner diameter, Kleiner AG, Switzerland) and purging of air in the system. All the sealed bags were submerged in a single reservoir to equalize hydrostatic pressure between solutions. The reservoir was situated about 10 cm above the microscope stage to allow gravity feeding of solutions. The Teflon lines were connected to valves which controlled the flow of solutions. At any time, a single solution was selected to flow through a Lucite manifold and into a common Teflon line which accessed a compartment of the chamber. The effluent from each compartment was collected, and measured at the end of an experiment to determine the average perfusion rate through a compartment. This value was used to determine the arrival time of different solutions, in conjunction with (1) dead volume measurements in the common Teflon lines and (2) times of valve switching. Perfusion was run at 500 to 900 μl/min. The $t_{1/2}$ of mixing (in all utilized portions of the chamber) was 6 to 7 sec in 165 μl at 500 μl/min, as measured by

the appearance or disappearance of fluorescent dyes in solution. Changes in the flow rate or compartment volume produced roughly proportional changes in the $t_{1/2}$ of mixing in the appropriate direction (*data not shown*).

Solutions

For most of the presented experiments, cells were placed either in a medium resembling culture medium in its ionic content ('Na^+ medium', containing in mM: 152 Na^+, 5 K^+, 2 Ca^{2+}, 1 Mg^{2+}, 1 SO_4^{2-}, 1 PO_4^-, 149 Cl^-, 25 glucose, 20 HEPES, pH 7.4 at 25°C) or in a medium with tetramethylammonium replacing Na^+ ('TMA^+ medium'). Also used was a 'K^+-clamp medium' containing in mM: 95 K^+, 27 Na^+, 22 TMA^+, 2 Ca^{2+}, 1 Mg^{2+}, 1 SO_4^{2-}, 1 PO_4^-, 136 Cl^-, 25 glucose, 20 HEPES, pH titrated from 6.0 to 8.0 at 25°C with TMA-OH. In order to evaluate the null point of K_i^+, a variety of solutions were used. They were based on a medium with K^+ completely replacing Na^+ in 'Na^+ medium'. This fully K^+-substituted medium was blended with TMA^+ medium and/or Na^+ medium to produce defined concentrations of K^+ using either TMA^+ or Na^+ as the replacement ion. A series of media (with varying K^+ and TMA^+) was also mixed so that 149 mM Cl^- was replaced with 75 mM gluconate and 74 mM Cl^-.

Microspectrofluorometry

Routinely, cells were loaded with BCECF (a fluorescent dye with a pH-sensitive spectrum; Rink et al., 1982) by exposure for 45 to 60 min to 1.25 μM (25 nmol total) of the acetoxymethyl ester (Molecular Probes, Oregon) at room temperature in Na^+ medium. After the dye-loading interval, cells were built into the chamber, mounted on the microscope and left for 10 min to re-equilibrate in Na^+ medium. This procedure resulted in levels of intracellular dye which were similar to those observed previously in suspensions of LLC-PK_1 cells (roughly 30 μM; Montrose & Murer, 1986). All experiments were performed at room temperature using solutions without added bicarbonate. Results from cells were accepted based on a homogeneous distribution of the dye in the cytosol both at the beginning and at the end of the experiment. In general, cells were selected which held dye relatively well during the re-equilibration period (prior to data collection). Cells in both the middle (14 cells) and the edge (19 cells) of the monolayer were used in the studies on glass coverslips, but cells in subconfluent patches were frequently disqualified because the dye was not distributed diffusely. Cells which were in the middle of a dome (on glass coverslips) were not commonly used because of apparent dye trapping beneath the dome. No other selection criteria were consciously applied. For experiments involving valinomycin, cells were dye-loaded in the presence of 9 μM valinomycin (Sigma) and subsequently perfused with 0.9 μM valinomycin. For experiments using nigericin, cells were dye-loaded in K^+-clamp medium at pH 7.4 or 6.0 which contained 3 μM nigericin (Calbiochem) and subsequently perfused with 0.3 μM nigericin. Under these conditions, exposing cells to increases in nigericin concentration in the perfusion solution produced no additional change in the apparent pH_i (*data not shown*). These exceptions to the routine protocol were used because introduction of these two ionophores via the perfusion system was slow without pre-exposure during dye loading.

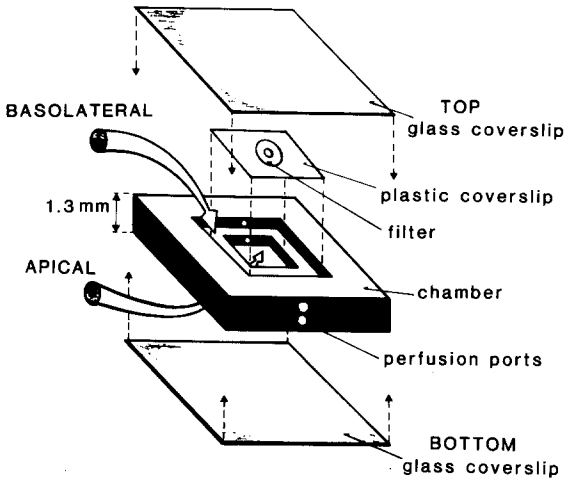

Fig. 1. A diagram of the stainless steel chamber which was used in the current studies. The system is shown for use with cells attached to a filterslip (*see* text) which was mounted allowing perfusion of the spaces both above ('basolateral') and below ('apical') the permeable support. Note that cells are attached to the side of the filterslip facing the bottom coverslip (and the objective of the inverted microscope)

A Zeiss microspectrofluorometry system (Carl Zeiss, West Germany) built around an IM35 (inverted) microscope was used in these studies. All fluorescent measurements were made in response to 7-msec pulses of light from a 100 W mercury lamp, whose light was further modified by a heat filter (BG 38) and a 0.5 neutral density filter. Intracellular pH was measured by forming a ratio of BCECF fluorescence calculated after sequential excitation of the dye using two bandpass filters: 390-440 nm and 475-490 nm. These selected wavelengths permit a wide dynamic range of observed ratios, since the sensitivity to pH in the BCECF excitation spectrum has an isobestic point near 440 nm (Tsien & Poenie, 1986). The excitation beam was reflected off a 510-nm dichroic mirror and directed through a 50× objective (Leitz) to the chamber. Epifluorescent emission from a defined section of the microscopic field was collected between 515 and 565 nm and quantified by a photomultiplier tube. The excitation filters were supplied by Oplabs (Lyngby, Denmark) and all other filters by Carl Zeiss. Under these conditions, fluorescein-labeled beads (Flow Cytometry Standards, North Carolina) bleached 0.12% per excitation pair, and the ratio of fluorescence changed very slightly (equivalent to 0.03 pH units) after 200 such pairs. No correction was applied to the data for effects due to bleaching.

The output from the photomultipler tube was amplified, digitally displayed, and manually put into a calculator which printed the time, raw data and calculated ratio (based on previously inputted values of autofluorescence). Optimally, the time resolution was 6 sec per measurement for the entire procedure, including time to manually position the filters, and to check position and focus of the microscopic field for each measurement.

Cells loaded with BCECF had fluorescent signals which were high compared to the background measured in cell-free regions of the chamber. In 30 BCECF-loaded cells on glass coverslips, the initial readings were 45 ± 5 (390-440 nm) and 135 ±

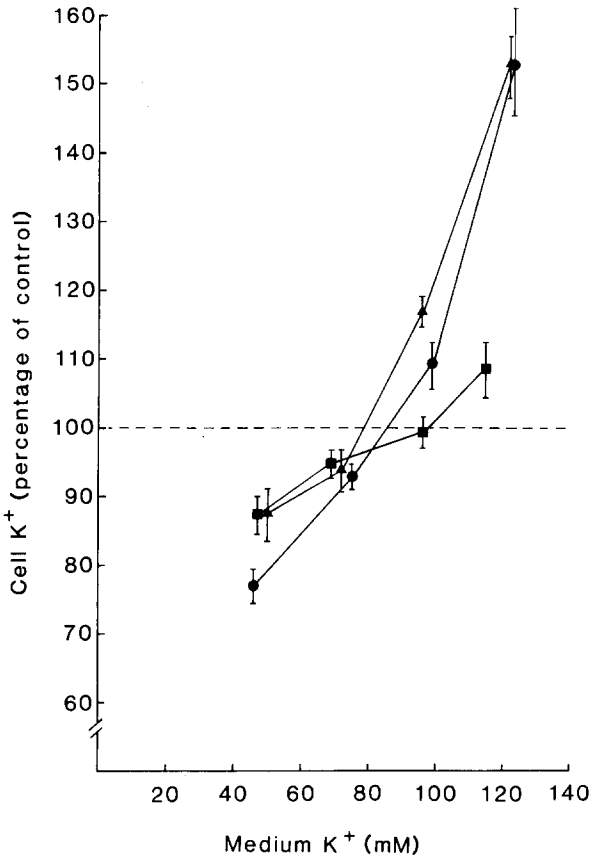

Fig. 2. A null point analysis of total cellular K^+. Cells remaining in culture medium (100% = 602 ± 36 nmol K^+/mg protein) were compared to cells incubated for 60 min at 37°C in isosmotic media containing the indicated concentrations of K^+. Medium K^+ concentrations were verified by flame photometry. Cellular K^+ levels were also determined by flame photometry, as described in Materials and Methods. The three curves present data with different ionic constituents in the medium (*see* Materials and Methods for details). In one curve (●), Na^+ was used as the replacement ion for K^+. In the other two curves, TMA^+ was used as the replacement ion for K^+ and other ions were kept at low and constant levels. In one case (▲), medium Na^+ was constant (27 mM) throughout the series. In the other case (■), Na^+ (27 mM), Cl^- (74 mM), and gluconate (75 mM) were constant in the media series. Mean ± SEM of four experiments are presented

15 (475-490 nm)-fold over this background. In 29 cells which were *not* loaded with BCECF, the cellular autofluorescence was 1.42 ± 0.2 (390-440 nm) and 1.46 ± 0.5 (475-490 nm)-fold over the same background. Under these conditions, only a small error is produced if the ratio is calculated using a cell-free background compared to the formally correct procedure of using values of cellular autofluorescence. In the 30 cells just described, a maximum error of 0.03 in the initial pH value is predicted based on the average cellular autofluorescence values described above. Therefore, when using cells on glass coverslips, working autofluorescence values were always taken from the edge of the coverslip in an area without cells. The autofluorescence of the (cell-free) filters was significantly higher than that of glass coverslips (roughly 10-fold higher with a 390-440 nm excitation) and eliminated the possibility to detect cellular autofluorescence on the substrate. For work with filterslips, two alternative techniques were found to give similar values of autofluorescence. In one technique, 100 μM digitonin (Calbiochem) were perfused into the chamber at the end of the experiment to release intracellular BCECF. In a second technique used routinely, measurements were taken prior to an experiment from a cell-free filterslip in a second chamber, which was filled with perfusion solution and mounted in the microscope (filter fluorescence was homogeneous and reproducible between filters).

Flame Photometry

The culture medium bathing cells on 35-mm dishes was replaced by experimental solutions for 60 min at 37°C. Total cellular K^+ was then determined as described previously (Montrose & Murer, 1986). Briefly, after experimental treatment, cells on 35-mm dishes were washed four times with ice-cold solution (containing in mM: 155 TMA^+, 3 Mg^{2+}, 1 SO_4^{2-}, 154 Cl^-, 1 EGTA, 10 HEPES, pH 7.4 when titrated at 25°C). Cells were then lysed in 1 ml 10% perchloric acid and the cell extract read directly in a Corning 410 flame photometer against standards in 10% perchloric acid. Results are presented either as a relative value or on a nmol/mg protein basis. Protein was determined by the Bradford procedure (Biorad) using gamma-globulin as standard.

Electron Microscopy

Cells on filterslips were prepared for electron microscopy by sequential dehydration in ethanol, embedding in Epon 812, and sectioning perpendicular to the plane of the filter. Sections were examined in a Philips 300 transmission electron microscope. Scanning electron microscopy was performed by critical point drying and gold coating of a filter followed by examination in a Philips SEM505.

Miscellaneous Techniques

Ethylisopropylamiloride (EIPA) was synthesized as described previously (Cragoe et al., 1967). EIPA was added to solutions from a 1-mM stock in DMSO, FCCP (Sigma) from a 1-mM stock in 50% ethanol, and valinomycin from a 4.5-mM stock in ethanol. All chemicals not specifically mentioned were purchased either from Sigma (St. Louis) or Fluka (Switzerland). Unless mentioned, data are presented either as mean ± SEM, or as a representative experiment.

Results

Method for Measurement of pH_i in Single Cells

In order to use BCECF to measure the intracellular pH (pH_i) of single cells in a microspectrofluorometer, it is first necessary to calibrate the fluorescent dye in the system. The best established technique for the intracellular calibration of dyes which sense pH is the method of Thomas et al. (1979). This tech-

nique uses nigericin (which catalyzes electroneutral K^+/H^+ exchange across cellular membranes) to bring pH_i to defined levels (a steady state between K^+ and H^+ gradients such that $K_i^+/K_o^+ = H_i^+/H_o^+$). This allows calibration of dye response versus pH without the release of dye from the cells. The use of the technique requires (1) knowledge of K_i^+ levels and (2) use of a saturating concentration of the ionophore (to assure that the condition of $K_i^+/K_o^+ = H_i^+/H_o^+$ is attained).

Flame photometry of total cellular K^+ has been used to produce an estimate of K_i^+ levels in LLC-PK_1 cells via a null point analysis. In Fig. 2, cells incubated in medium with valinomycin and different K^+ concentrations are compared to (control) cells left in culture medium. As shown in Fig. 2, in the presence of valinomycin, the cellular K^+ levels detected by flame photometry are altered after exposure to different concentrations of K^+ in the medium. Valinomycin was used to produce a defined K^+ permeability in the cell membrane, but increases the problem of alterations in cellular K^+ levels due to electrogenic movement of the other major ions in the medium. For example, electrogenic flux of Na^+ down its electrochemical gradient will cause compensatory movement of the most permeant alternate ion (K^+ efflux from the cell). In order to evaluate the magnitude of the effect of Na^+ and Cl^- gradients on K^+ movement, null point measurements were performed using either (1) a simple substitution of Na^+ for K^+, or (2) a substitution of TMA^+ for K^+ where in addition, Cl^- and/or Na^+ concentrations in the medium were held constant at lower concentrations than in the former protocol (75 and 27 mM, respectively; with gluconate replacing Cl^-). As shown in Fig. 3, the measured null point of cellular K^+ shifts between 80 and 95 mM under these different conditions. Control experiments indicated that the altered steady-state K^+ levels were attained by 20 min and that the addition of 100 μM ouabain had no effect on the new steady-state level (*data not shown*). The value of 95 mM was obtained in a protocol where electrogenic fluxes of Na^+ and Cl^- should be reduced (low medium Na^+ and Cl^-), and therefore has been used in further work as the best estimate of K_i^+. In order to accept this value as an estimate of K_i^+, one must assume that the flux of K^+ in the presence of valinomycin is larger than other routes of net K^+ flux, and that with 95 mM medium K^+, the membrane potential (E_m) is near zero. These assumptions may be reasonable since no effect of flux via the Na^+,K^+-ATPase could be detected, and since similar concentrations of valinomycin produce a K^+ permeability which is much higher relative to the intrinsic permeabilities of other ions in an epithelial cell (Kimmich et al., 1985).

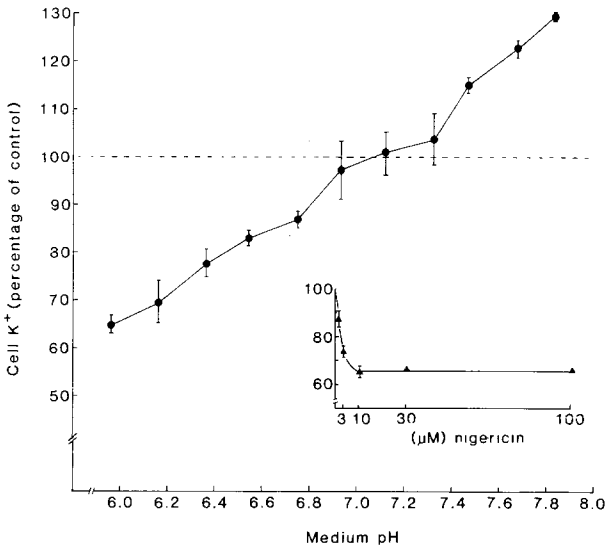

Fig. 3. Effect of nigericin on total cellular K^+ levels. Cells remaining in culture medium (100% = 628 ± 28 nmol K^+/mg protein) were compared to cells incubated for 60 min at 37°C in K^+-clamp medium of varying pH (95 mM K^+, 27 mM Na^+; *see* Materials and Methods). In the results presented in the inset, cells were placed in K^+-clamp medium at pH 6.0 (60 min, 37°C) with the indicated nigericin concentrations. The *y*-axis of the inset is the same as the main Figure. Means ± SEM of four experiments are presented

It was determined whether the estimate of K_i^+ was reasonable by observing changes in total cellular K^+ levels which were induced by nigericin and alterations in medium pH. In Fig. 3, the experimental levels of cellular K^+ are compared to (control) cells left in growth medium. As shown in the Figure, in the presence of 10 μM nigericin and a constant K^+ concentration (95 mM) in the medium, the cellular K^+ levels are significantly affected by alterations in medium pH. This is predicted to occur due to the net K^+ for proton exchange which is catalyzed by nigericin in order to reach a steady state such that $K_i^+/K_o^+ = H_i^+/H_o^+$. The results in the inset of Fig. 3 indicate that with medium pH 6.0, 10 μM nigericin produces a saturated effect in the system. Control experiments indicated that new steady-state K^+ levels were attained within 30 min in the presence of 10 μM nigericin (*data not shown*).

If 95 mM is a correct value for K_i, then the crossover point in Fig. 3 is an estimate of resting pH_i in the cells at the time when they are exposed to the nigericin-induced pH_i clamp (i.e. cells in culture medium). If a K^+-clamp medium was used in which CO_2 was maintained at the same level as in the culture medium (10%) throughout the experiment, then at medium pH 7.0, cellular K^+ was 94 ± 4 percent of control in four experiments. This suggests that removal of CO_2 has only a small effect on the deter-

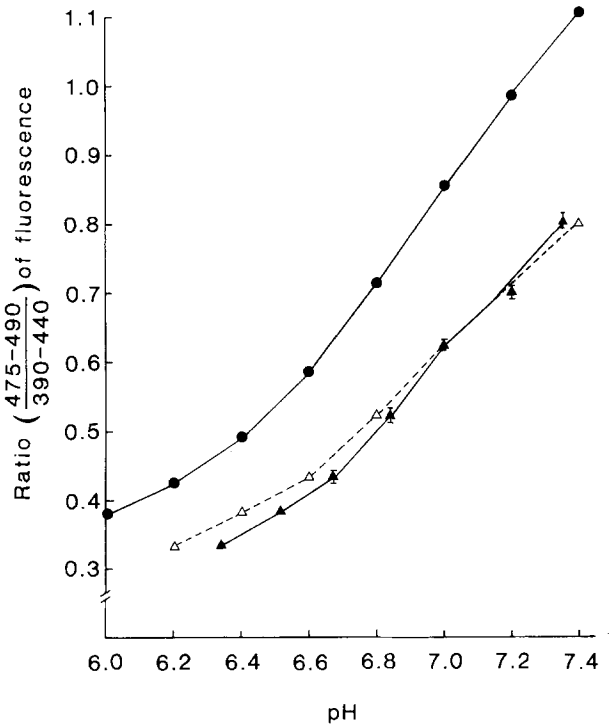

Fig. 4. Calibration curves of BCECF measured in the microspectrofluorometer. In all presented data, a ratio of fluorescence was calculated due to sequential excitation of the dye with light of 390-440 nm, then 475-490 nm. In one curve (●), solutions of unesterified BCECF (1 μM) were placed on glass coverslips and measured on the microscope stage. In the other curves (△, ▲), intracellular dye was calibrated in single cells on glass coverslips by exposure of the cells to saturating concentrations of nigericin in K^+-clamp medium of varying pH. One of these curves (△) presents the data versus medium pH. The other (▲) presents the same data corrected for changes in intracellular K^+ levels due to nigericin. Mean ± SEM of 15 determinations (or 15 cells) are presented

mination of pH_i in these results. In these experiments, medium Na^+ was kept constant at 27 mM to approximate intracellular Na^+ levels (Montrose & Murer, 1986), and therefore also minimize shifting of the crossover due to Na^+ transport by nigericin. The crossover value which is observed (approximately pH 7.1) is similar to that observed previously in suspensions of LLC-PK_1 cells (resting pH_i = 7.05), which supports the assumption that 95 mM is a reasonable estimate of K_i.

Using this supporting information, the response of intracellular BCECF to defined changes in pH_i could be evaluated. Two calibration curves are presented for comparison in Fig. 4. They both present the sensitivity of the experimentally measured parameter on the microscope (a ratio of fluorescence due to different excitation wavelengths) versus pH. The upper curve was generated using solutions of (1 μM) unesterified BCECF placed on a coverslip on the microscope stage. For this curve, the pH scale of the *x*-axis refers to the solution pH. The lower curves present results from cells on glass coverslips which were loaded with BCECF and pH-clamped using nigericin. Cells were exposed to saturating concentrations of nigericin in the perfusion system (*see* Materials and Methods) and then were progressively exposed to media of different pH, from a starting value of 7.4 (with time to achieve a new steady state of ratio between steps). In the Figure, the open triangles present ratio versus medium pH for these experiments. The filled triangles are the same data after correction for the alterations in K^+ produced by nigericin. That is, with medium pH at 6.2, the cellular K^+ (*see* Fig. 3) is reduced to 70% of the starting value, and therefore since $K_i^+/K_o^+ = 70/100$, nigericin should cause $H_i^+/H_o^+ = 0.7$. As shown, the correction is quite small unless pH_i values are encountered which are extremely far away from resting pH_i. This was predicted by Chaillet and Boron (1985). The values presented in the filled triangles were considered the best available calibration for ratio versus pH_i and were used in all subsequent experiments with that particular mercury lamp. This seemed justified because the variation between cells in the presence of the nigericin clamp was very small (note the standard error bars in Fig. 4), indicating that the dye behaved similarly in all measured cells. In addition, nonsystematic checks of the unesterified dye calibration curve (*data not shown*) indicated that the lamp remains stable throughout its lifetime.

Mechanism of pH_i Recovery: LLC-PK_1 Cells on Glass Coverslips

LLC-PK_1 cells were grown on glass coverslips and single cells were examined in the microspectrofluorometer for the mechanism of pH_i recovery from an acid load. Cells loaded with BCECF were built into the chamber diagrammed in Fig. 1 and described in Materials and Methods. After a period of re-equilibration (*also see* Materials and Methods), a cell was selected and the experiment started.

When the LLC-PK_1 cells are first mounted in the microscopy chamber, the observed fluorescence ratio of most of the cells is quite high (corresponding to pH 7.62 ± 0.06, 18 cells). This is consistent with the removal of cells from a medium containing CO_2 (a weak acid), but could have another basis as well. As shown previously for LLC-PK_1 and other cell types, a NH_4 prepulse (NH_4Cl preincubation and removal) results in an intracellular acid load (Roos & Boron, 1981; Montrose & Murer, 1986).

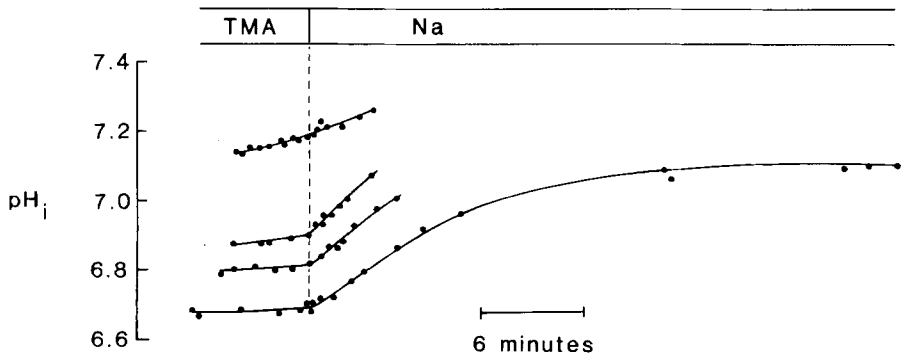

Fig. 5. Response of an LLC-PK_1 cell to progressively larger acid loads. Cells grown on a glass coverslip were mounted on the microscope and allowed to re-equilibrate under perfusion (*see* Materials and Methods). A cell with homogeneous, diffuse fluorescence was selected, and experiments started, after this interval. The cell under study was subjected to four repeated acid loads due to NH_4-prepulse (20 mM NH_4 in TMA^+ medium). When NH_4 was removed to initiate the acid load, the cell was maintained in TMA^+ medium (TMA^+). The Figure presents the results after pH_i has reached a minimum following NH_4 removal. Each set of data points represents the results after an acid load, with the cell brought to more acidic pH_i after every round of NH_4 pulsing. The results after each acid load have been vertically aligned (with respect to the switch to Na^+ medium (Na^+) as the perfusate), to allow a comparison of Na^+-independent and Na^+-dependent pH_i changes. The arrival time of solutions was calculated as described in Materials and Methods and the observed ratio of fluorescence was converted to pH_i values (in this and all subsequent Figures) using the calibration curve in the previous Figure. Lines were fit to the data by eye

Several such NH_4 prepulses could be used in series to produce a progressive acidification of the cell under study, relative to the starting value. The presentation of results in Fig. 5 is the result of a series of four NH_4 prepulses which resulted in a larger acid load (lower pH_i) after the completion of each prepulse. Only the data after removal of NH_4 is shown. The results have been 'stacked' (without a continuous time base) to allow a visual comparison of the results in each case before and after the addition of Na^+ to the perfusate. As shown in the Figure, the relative acidification due to the first NH_4 prepulse (results at top of Figure) does not hold constant, and this Na^+-independent pH_i change is relatively insensitive to the substitution of Na^+ medium as the perfusate. After reacidification to a lower pH_i by another NH_4 prepulse (second trace from the top), a Na^+-dependent component of pH_i recovery is observed. Notice that in this and the two subsequent pH_i recoveries (lower traces), the Na^+-dependent component (of pH_i change/min) is approximately constant, and the Na^+-independent component decreases at lower pH_i. Qualitatively similar results were observed in seven cells. The constancy of the Na^+-dependent pH_i change/min is similar to that observed previously in LLC-PK_1 cell suspensions when BCECF was used to detect Na^+/H^+ exchange capacity at different pH_i values (Montrose & Murer, 1986).

The results presented in Fig. 5 allow the identification of a pH_i 'set point' at which the cell becomes more sensitive to the readdition of Na^+ versus TMA^+. The set point may be observed either as a pH_i range over which the Na^+-dependent pH_i recovery 'appears' (pH_i 6.9 to 7.2 in Fig. 5), or as the steady-state pH_i to which the cell recovers after an acid load (pH 7.1 in Fig. 5). It is not known with certainty that the Na^+-independent pH_i change is altered after the addition of Na^+, but the cells were always observed to reach a steady-state pH_i which was more stable than predicted by the Na^+-independent component at that pH_i (as shown in Fig. 5). Despite the fact that all cells examined demonstrate a Na^+-dependent component of pH_i recovery, it was noted that it was impossible to predict the exact steady-state pH_i which the cells would reach or at which the Na^+ dependence would appear. With these two criteria, steady-state pH_i values between pH 6.83 and 7.38 were noted, with an average resting pH_i of 7.09 ± 0.05 (standard deviation = 0.18) in 14 cells grown on glass coverslips. It is not known if the variability relates to differences in cell status, cell type (our cell line is not cloned), or system artifacts. Applying a (least-squares fit) line to the calibration curve between pH_i 6.7 to 7.4, the apparent variability of 15 cells which were pH clamped with nigericin at pH 7.0 is smaller (pH_i 7.03 ± 0.02; standard deviation = 0.07; range, pH 6.89 to 7.14).

The observation of a 'set point' for Na^+-dependent pH_i recovery from an acid load suggests that Na^+/H^+ exchange may be responsible for the pH_i change, since many laboratories have observed a change in capacity of Na^+/H^+ exchange with alterations in pH_i (Aronson et al., 1982; Grinstein & Rothstein, 1986; Montrose & Murer, 1986). Therefore we have examined the amiloride (derivative) sensi-

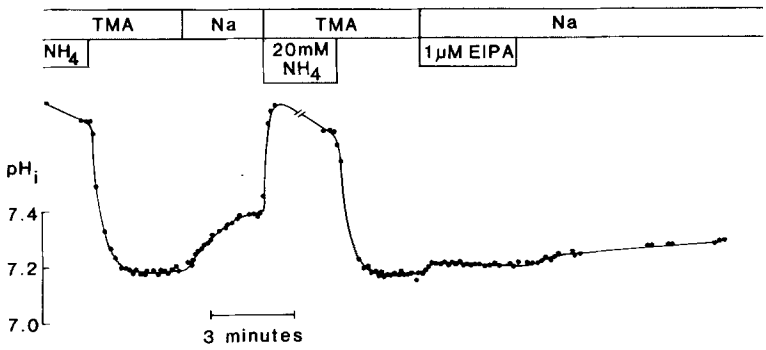

Fig. 6. Cellular recovery from an acid load is Na^+ dependent and sensitive to EIPA. A single cell on a glass coverslip was perfused with either TMA^+ medium (TMA^+), Na^+ medium (Na^+), or one of these solutions plus either 20 mM NH_4Cl (NH_4), or 1 μM EIPA as indicated in the Figure. In all six cells which were examined qualitatively similar EIPA inhibition was observed. Na^+-dependent recovery was observed in a total of 16 cells

tivity of the pH_i recovery. The presentation of results in Fig. 6 starts at a time after a series of NH_4 prepulses have been imposed on the cell. As shown in the Figure, the relative acidification after the first (presented) NH_4 prepulse is only relieved after substituted of Na^+ medium as the perfusate. After reacidification to the same level of acid load by another NH_4 prepulse, the Na^+-dependent pH_i change is shown to be decreased in the presence of 1 μM ethylisopropylamiloride (EIPA) in the perfusate. This decrease in the pH_i recovery probably represents inhibition of the process since (as shown in Figs. 5 and 11) repeated acid loads (in the absence of EIPA) only affect the rate of recovery slightly. This inhibition due to EIPA is poorly reversible, as shown in the Figure after removal of the inhibitor. The EIPA inhibition was observed in all six experiments of this type which were performed and suggests that Na^+/H^+ exchange may be responsible for the pH_i recovery. A comparison of Figs. 5 and 6 reveals that the rate of pH_i recovery is not constant between individual cells. This variability was not examined in detail, but could relate to individual differences in cellular buffering capacity, cell status, or expression of transport systems.

In order to more firmly define the mechanism of Na^+-dependent recovery from an acid load, LLC-PK_1 cells were examined for the presence of proton conductance. It has been shown previously that addition of FCCP to suspensions of LLC-PK_1 cells in Na^+ medium causes a cellular acidification. This was interpreted as an alignment of pH_i with E_m due to the presence of FCCP (and consequent proton conductance). In the current work we have observed the same phenomenon in single cells (*data not shown*) and have devised an alternate protocol in order to compare the sensitivity of the resting pH_i to changes in E_m, before and after FCCP addition. In this experiment, we seek to change E_m by changing medium K^+ in the presence of valinomycin. Thus the results presented in Fig. 7 were collected from a preparation in which valinomycin was present throughout the experiment (*see* Materials and Methods). Control experiments indicated that cells on plastic petri dishes had only slightly altered total cellular K^+ and Na^+ levels after 2 hr of (25°C) exposure to 9 μM valinomycin when compared to control cells without valinomycin (103 ± 9 and 155 ± 60 percent of control K^+ and Na^+, respectively, in three experiments; mean ± SD). This suggests that the cells in valinomycin could maintain an E_m close to control values. The cell under study in Fig. 7 was first acidified such that it demonstrated a recovery from an (NH_4 prepulse) acid load, to indicate that the cell had (at least temporarily) activated the pH_i-recovery mechanism. When the cell was then exposed to high K^+ medium (95 mM K^+, 27 mM Na^+), no significant alteration in pH_i was observed. This result is consistent with previous data (Montrose & Murer, 1986) indicating negligible intrinsic proton conductance and an insensitivity of Na^+/H^+ exchange to the Na^+ gradient at resting pH_i (probably due to kinetic inactivation of net transport via a pH_i 'set point'). In Fig. 7, the subsequent addition of FCCP to the high K^+ perfusate also produced minimal changes in pH_i (in two cells out of eight, pH_i rose at this stage in the experiment). If pH_i is not equal to medium pH (7.4) and E_m is exactly zero, then pH_i is predicted to always go towards 7.4 in these circumstances. Our results probably indicate that E_m is not exactly zero (e.g., due to a contribution of FCCP-induced proton fluxes to E_m). The next step in the Figure is to maintain the FCCP concentration, but replace the high K^+ perfusate with one which should make E_m more negative (Na^+ medium with 5 mM K^+). As shown, this transition causes a rapid acidification which may be reversed by readdition of the high K^+ medium. These last results are a sharp contrast to what is observed in the absence of FCCP when medium K^+ is changed. The results suggest that (1) changes in medium K^+ effectively change E_m (as evidenced by the observed effects in the presence of FCCP) and therefore (2) prior to FCCP addition, pH_i is unaffected by these alterations in E_m (low intrinsic proton conductance).

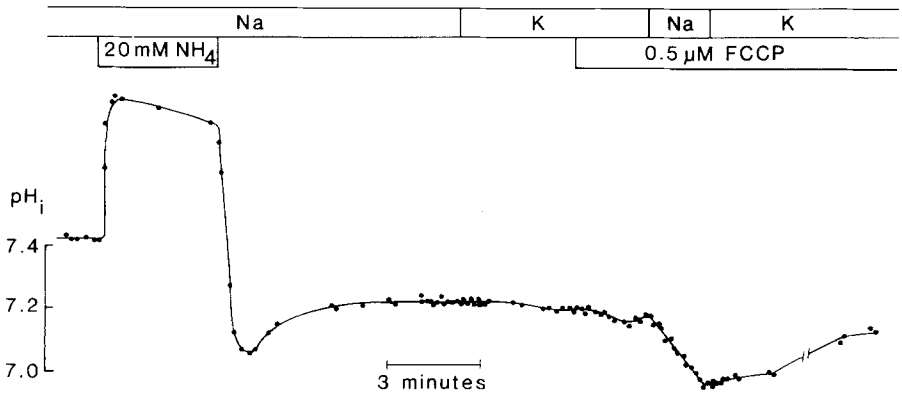

Fig. 7. Effect of changes in the membrane potential on resting pH_i before and after the addition of FCCP. The entire experiment was performed in the presence of valinomycin (0.9 μM in the perfusate solutions; *see* Materials and Methods). After the experimental cell on the coverslip was acidified sufficiently to demonstrate a pH_i recovery (as shown in the Figure), it was alternatively exposed to either Na^+ medium or K^+-clamp medium (pH 7.4 denoted K^+). FCCP (0.5 μM) was added in the indicated perfusates. The break in the line which is drawn through the data points represents a gap of 5 min. Qualitatively similar results were obtained in eight cells

Mechanism of pH_i Recovery: LLC-PK_1 Cells on Filterslips

In order to determine whether the pH regulatory Na^+/H^+ exchange of LLC-PK_1 is expressed in a restricted membrane domain, we developed procedures whereby cells attached to a permeable support could be examined in the microspectrofluorometer. The procedure used to attach Nucleopore filters to a plastic coverslip (a construction referred to as a filterslip) requires exposure to acetone. However, the scanning electron micrograph in Fig. 8*C* shows that while the diameters of the pores in the filter are increased by this procedure, the filter is still intact. As shown in Figs. 8*A* and 8*B*, cells on (collagen-treated) filterslips have normal morphology and polarity (note the brush-border and tight junctions) as viewed in the transmission electron microscope. The collagen treatment probably produces a single layer of adhered monomers which are not well visualized in the microscope.

Cells on filterslips were mounted in a dually perfused chamber, as shown in Fig. 1, and examined for the requirements for recovery from an acid load. Several criteria were used to determine the membrane location of the pH-regulatory Na^+/H^+ exchange. In all protocols, it was first determined that both apical and basolateral addition of NH_4 were capable of independently alkalinizing the cell under study. This comprised a 'local accessibility' control which had to be satisfied prior to continuence of the experiment on that cell. A similar control for 'local tightness' was not performed, but was not crucial since most experiments demonstrated a sidedness of pH_i recovery from the apical versus basolateral solutions.

The results in Fig. 9 show that in one protocol, the addition of basolateral Na^+ is required to initiate a rapid recovery from an acid load. As described above, the subsequent application of apical NH_4 was a convenient way to indicate that there were no problems in the perfusion system or with accessibility of apical solutions to the particular cell under investigation. In 16 cells, Na^+ in the basolateral perfusate caused at least a 10-fold increase of pH_i change per minute. The results suggest that basolateral Na^+/H^+ exchange is responsible for pH regulation under these conditions.

The results in Fig. 10 present another protocol which examines the effect of removal of basolateral Na^+ after initiation of a pH_i recovery. As shown in the Figure, despite the continued presence of apical Na^+, the removal of basolateral Na^+ causes reversal of the pH_i recovery. This could represent reversal of the Na^+/H^+ exchange reaction in the basolateral membrane if intracellular Na^+ levels are high enough so that the outwardly directed Na^+ gradient is greater than the proton gradient. The observation of a reversal of pH_i recovery is not easily explained in the presence of significant Na^+/H^+ exchange in the apical membrane, as compared to that in the basolateral membrane. Note that while the time of arrival of basolateral Na^+ coincides with initiation of the pH_i recovery, the removal of Na^+ takes longer to initiate the reversal phase. This was noted in six experiments. The difference probably relates to the perfusion system, which (with a $t_{1/2}$ of mixing of 6 sec) can introduce Na^+ quickly at an effective concentration (75 mM in 6 sec), but can only remove it to low concentrations more slowly (15 mM in 30 sec).

A third protocol could also be used to suggest

that Na^+ was required at the basolateral membrane for pH_i recovery, even when the monolayer was not ideally tight. In some preparations (as shown in Fig. 11), the addition of apical Na^+ clearly initiated a pH_i recovery which was further increased by adding basolateral Na^+. However, when the cell was reacidified, a reversal of the order of addition demonstrated that addition of basolateral Na^+ evoked the full rate of recovery without any significant increase due to subsequent addition of apical Na^+. The data suggest that limited cross contamination of the apical and basolateral solutions is occurring, but that only Na^+ spillover into the basolateral compartment is important in altering the pH_i recovery. Assuming a single type of Na^+/H^+ exchanger and a symmetrical cross contamination (at the cell surface), the

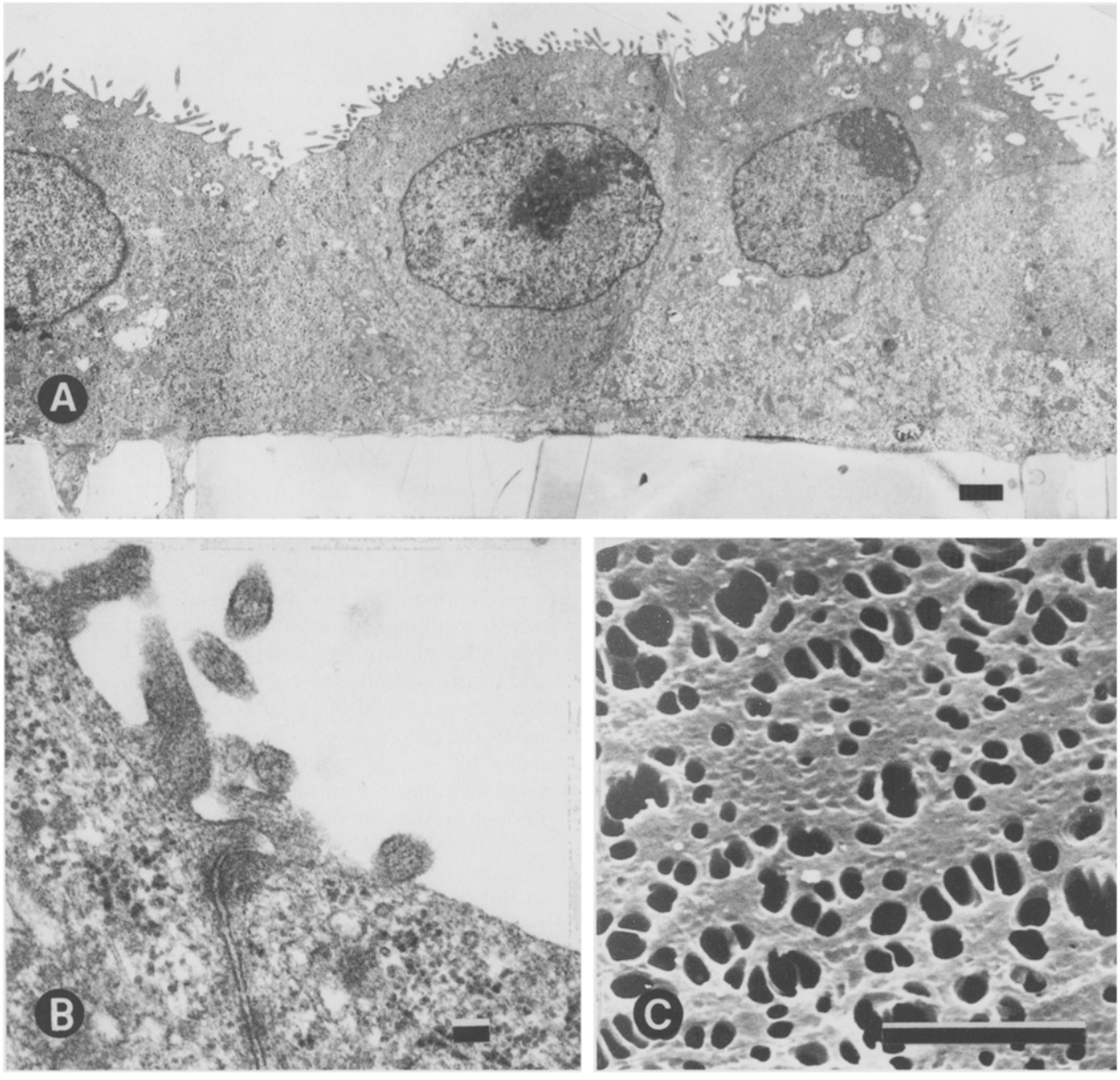

Fig. 8. Electron microscopy of a filterslip and the cells grown on filterslips. (*A*) Transmission electron micrograph of LLC-PK_1 cells seeded at confluent density onto a colllagen-treated filterslip, then prepared for electron microscopy the following day. The bar is a 1-μM scale. These cells were in the section of the filter which was over the hole in the plastic coverslip. Note the filter which is still visible beneath the cells and the presence of apical micovilli on the cells. (*B*) A high magnification micrograph of a tight junction between cells grown as in (*A*). The bar is a 0.1-μm scale. (*C*) Scanning electron micrograph of 0.8-μm pore Nucleopore filter that was constructed for use as a filterslip. The section of the filter which is shown was the portion over the hole in the plastic coverslip. The bar is a 10 μm scale

data imply that basolateral Na^+/H^+ exchange is responsible for the pH_i recovery which is observed.

Data was compiled using the three different criteria described above to define the results observed in LLC-PK_1 cells. As shown in the Table, the overwhelming number of cells in monolayers which demonstrate sidedness (defined as some observable difference in the rate of recovery due to apical versus basolateral Na^+) also demonstrate basolateral Na^+/H^+ exchange as the major mechanism of recovery from an acid load. The data in the Table also present values for a series of experiments using two different conditions of cell seeding density and collagen pretreatment of filterslips. The results presented in Figs. 9 to 12 present examples of the results obtained with collagen treatment and different seeding density. As shown in the Table, neither of these parameters affected the results. In 13 cells on filterslips which fully recovered from an acid load, the average value of resting pH_i was 7.11 ± 0.06.

In order to present evidence that the Na^+-dependent pH_i recovery observed in cells on filterslips was due to Na^+/H^+ exchange, the EIPA-sensitivity of the pH_i recovery was examined. Figure 12 presents data from cells grown on a filterslip and perfused with TMA^+ medium continuously in the apical compartment. The NH_4 prepulses which

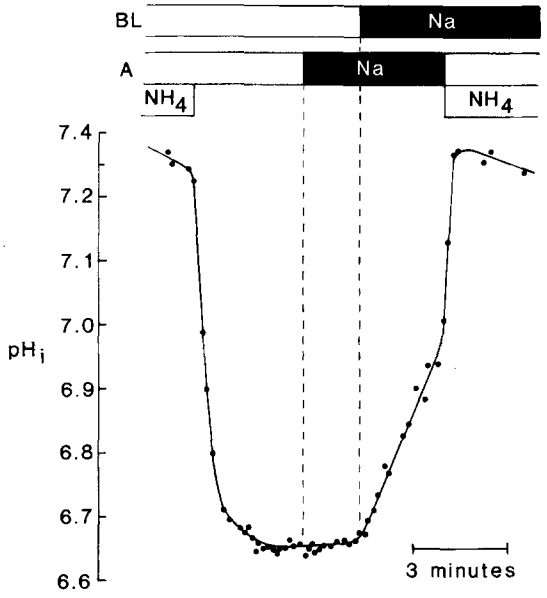

Fig. 9. Effect of apical versus basolateral addition of Na^+ on the initiation of pH_i recovery from an acid load. Cells were seeded below confluent density (*see* Materials and Methods) onto a collagen-treated filterslip. The filterslip was built into a dually perfused chamber to allow independent access of the perfusates to the apical (*A*) and basolateral (*BL*) membranes of the cells. The cell under study was perfused with either Na^+ medium (Na^+, in black sections) or TMA^+ medium (white sections). This graphical procedure for highlighting the addition of Na^+ is followed in all the remaining Figures. NH_4Cl (20 mM) was present in (apical) TMA^+ medium at the indicated times. Similar results were observed in 16 cells

Table. Effect of seeding density and collagen treatment on the number of cells with a certain polarized status for Na/H exchange[a]

	Number of cells in polarized status		
	Apical	Basolateral	Not determined
Seeding density:			
confluent	1	17	4
subconfluent	0	5	0
Filterslip pretreatment:			
none	0	5	1
collagen	1	17	3
Total cells studied	1	22	4

[a] The criteria described in the text were used to evaluate the membrane location the Na/H exchanger using 27 cells. Conditions which are outlined above are described in detail in Materials and Methods. When cells did not demonstrate any sidedness in the preparation (judged by Na addition), then they were placed in the 'not determined' category.

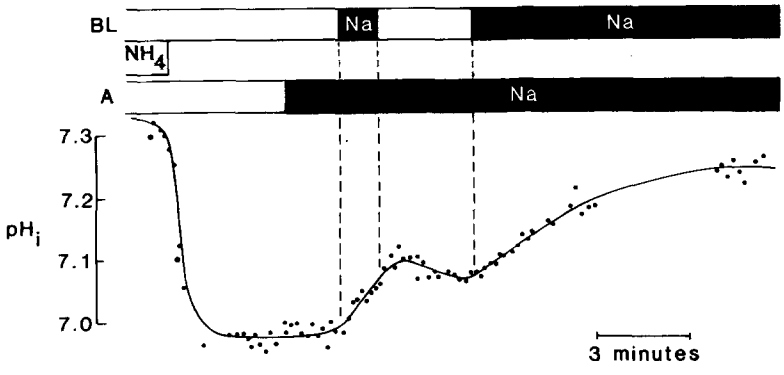

Fig. 10. Demonstration of reversibility of pH_i recovery due to basolateral Na^+. In this experiment, the cells were seeded at confluent density onto collagen-treated filterslips. As shown, the removal of Na^+ from the basolateral compartment causes the cell under study to halt, and then reverse, the pH_i recovery which had been previously initiated by the addition of Na^+ to the basolateral compartment. Similar results were observed in six cells

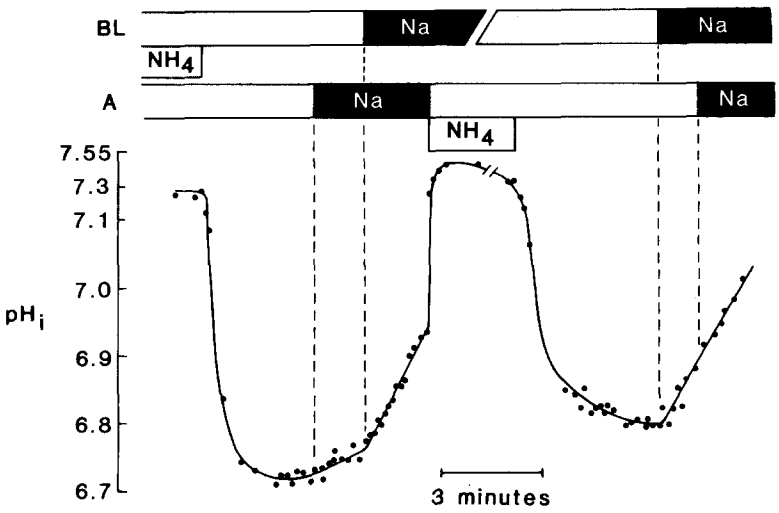

Fig. 11. Effect of ordered additions of Na^+ from the apical and basolateral compartments on pH_i recovery from an acid load. In this experiment, the cells were seeded below confluent density onto collagen-treated filterslips. As shown, the addition of apical Na^+ alone is sufficient to initiate a pH_i recovery from an acid load. This recovery is stimulated by further addition of basolateral Na^+. When the cell is reacidified, and the order of Na^+ addition is reversed, the basolateral Na^+ initiates a pH_i recovery similar in rate to the maximal rate observed previously. Further addition of apical Na^+ causes no detectable increase in the pH_i recovery. Similar results were observed in six cells. The gap in the data represents 7 min

were used to produce the cellular acid load were omitted from the Figure. As shown in the Figure, addition of 0.1 μM EIPA to the basolateral perfusate caused a significant decrease in the rate of pH_i recovery due to Na^+. Similar results were obtained in five preparations. The results in Fig. 12 also indicate that the inhibition is weakly reversible after removal of the inhibitor, as shown previously in Fig. 3. We conclude that in most LLC-PK_1 cells which were examined on filterslips basolateral Na^+/H^+ exchange is the major mechanism responsible for pH_i recovery from an acid load.

Discussion

The current work introduces a microspectrofluorometry system and uses the system with LLC-PK_1 cells to evaluate the recovery of pH_i from an intracellular acid load. These cultured cells were chosen because of previous results which have shown that the cells express both enzymatic and membrane transport proteins in restricted membrane domains (apical or basolateral membranes) (Rabito, 1981; Rabito & Karlish, 1983; Gstraunthaler et al., 1985). In addition, the cells show morphological polarization with respect to the development of tight junctions and microvilli. These features are all consistent with a renal epithelial origin of the cells. In addition, several laboratories have observed Na^+/H^+ exchange in the LLC-PK_1 cell membrane either in cell monolayers (Haggerty et al., 1985; Cantiello et al., 1986; Chaillet et al., 1986), isolated membranes from the cells (Moran et al., 1986), or in suspensions of cells. In suspensions of LLC-PK_1 cells, Na^+/H^+ exchange was found to be a major regulator of resting pH_i (Montrose & Murer, 1986).

The introduction of the microspectrofluorometry system mandated a calibration of the fluorescence of intracellular BCECF versus pH_i. In these experiments, a ratio of fluorescence at two different excitation wavelengths was used to characterize the response of the dye to changes in pH. Similar procedures have been used successfully in other systems to provide a value virtually independent of dye concentration and dye bleaching (Chaillet et al., 1985; 1986). When pH_i was drawn far from resting pH_i by saturating concentrations of nigericin, alterations in K_i^+ were taken into account in the calibration of dye response versus pH_i. This correction was only slight, as predicted by Chaillet et al. (1985). The intracellular calibration curve was significantly different from that of unesterified BCECF in solution (also measured on the microscope stage). This can relate to many factors including differences in optical geometry, alterations in the pK_a of BCECF in the intracellular compartment, or the fluorescence ratio scaling factor which is introduced in the use of the ratio technique (Grynkiewicz et al., 1985).

Assuming that the estimate of (95 mM) K_i^+ is reasonable, the data in Fig. 3 give an initial estimate of average resting pH_i levels in the LLC-PK_i cells attached to a substrate (pH 7.1). This estimate is valid for the cells remaining in culture medium at 37°C, since this is the condition the cells were in directly before exposure to the pH clamp with nigericin. Despite the increase in pH_i which is observed in cells (without a pH clamp) after removal from medium containing CO_2, the pH-clamped cells seem to be relatively insensitive to the net proton loss induced by CO_2 removal. The pH_i estimate from the K_i^+ crossover was similar to that observed previously in suspensions of the same cells (pH 7.05; Montrose & Murer, 1986). The similarity suggests that the same mechanism of pH_i regulation

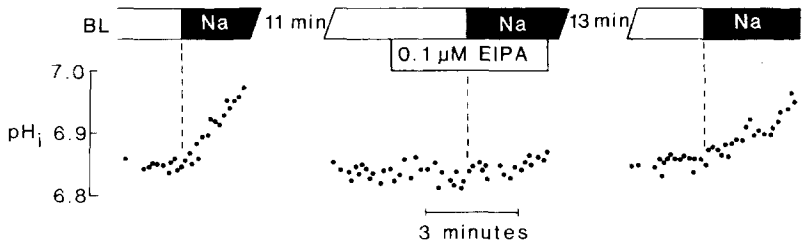

Fig. 12. Effect of EIPA on the pH_i recovery observed in cells on a filterslip. Cells were seeded onto collagen-treated filterslips at confluent density. TMA^+ medium was perfused continuously in the apical compartment during this experiment. The information collected during pH_i transients due to NH_4 prepulsing are not presented. Similar results were observed in five cells

may be in effect in both suspended cells and substrate-attached cells in culture. This suggestion is supported by the current evidence which identifies Na^+/H^+ exchange as an important pH-regulatory transport system in substrate-attached cells. In addition, a similar average resting pH_i has been observed for cells on glass coverslips or filterslips using BCECF. This information is supportive of the measurements in single cells being representative of the general population, although it is not known with certainty whether the selected cells represent a functionally distinct subpopulation in this uncloned cell line. Some variability and/or bias might result from the selection procedure which we have employed (as described in Materials and Methods).

The results obtained with cells on both glass coverslips and filterslips support the results obtained previously from suspended cells, with respect to a Na^+-dependent recovery from an acid load and the amiloride (derivative) sensitivity of this process. EIPA has been shown previously to be a potent inhibitor of Na^+/H^+ exchange in the LLC-PK_1 cell (Haggerty et al., 1985). EIPA (versus the parent compound, amiloride) was used in the current work because amiloride caused a significant alteration in the fluorescence ratio of cells which were pH clamped with nigericin (*data not shown*). Alterations in fluorescence ratio due to EIPA were negligible as long as BCECF fluorescence was not very low. The results in Fig. 12 indicate directly that addition of EIPA (added prior to Na^+ perfusion) had no detectable effect on the apparent pH_i.

The results from cells on glass coverslips also support the previous conjecture (Montrose & Murer, 1986) that sustained proton conductance in the plasma membrane is not responsible for setting the resting pH_i of the cells. In the current work changes in the K^+ gradient (with valinomycin) only altered the resting pH_i of the cells after addition of FCCP. Assuming that the changes in the K^+ gradient affect pH_i via changes in E_m, this implies that at resting pH_i, the cells have no detectable proton conductance (or other membrane potential-sensitive proton transport systems) active in the membrane. It is worth noting that any alterations in the membrane potential which were produced (due to changes in the K^+ gradient) may be smaller after the addition of FCCP since high proton permeability may act to partially clamp the membrane potential. The results suggest that transport events which simply bring protons to electrochemical equilibrium are not responsible for establishing the observed resting pH_i. Because of the 1 : 1 stoichiometry of $Na^+ : H^+$ net fluxes which have been observed previously (Montrose & Murer, 1986), and the amiloride (derivative) sensitivity of the pH_i recovery, our working hypothesis is that electroneutral Na^+/H^+ exchange is responsible for the pH_i recovery from an acid load.

The criteria which were used to evaluate the location of the Na^+/H^+ exchanger are based on the ability to define the arrival time, and the rapid effects, of additions to either the apical or basolateral compartments. The most conclusive results were obtained from preparations which demonstrated either small (or undetectable) rates of pH_i recovery due to apical Na^+, or reversible pH_i recovery upon removal of basolateral Na^+. However, by ordered additions, it was still possible to suggest that Na^+ was required at the basolateral membrane for the pH_i recovery in some less "tight" monolayers that nonetheless demonstrated some sidedness. In other preparations which appeared suitable in epifluorescent images, no sidedness was observed and these must represent cases where the perfusion solutions were not well separated. In these latter preparations, no conclusions regarding the cells could be made. The reasons for the occasional failure of the preparation may be a poorly built chamber or an incomplete (or damaged) monolayer.

The observed lack of Na^+/H^+ exchange in the apical membrane of the LLC-PK_1 cells is contrary to two previous reports. In previous experiments from our laboratory, membrane vesicles enriched in the enzyme marker trehalase were shown to catalyze Na^+/H^+ exchange (Moran et al., 1986). It has also been observed that in cells grown on collagen (fiber) coated Nucleopore filters, exposure to amiloride inhibited Na^+ influx from the apical solution (Cantiello et al., 1986). The explanation for these differences is not clear, but could relate to differences in the experimental assay conditions, hormo-

nal status of the cells, and/or substrate attachment conditions. Similar explanations are necessary to explain how it is possible to measure significant Na^+/H^+ exchange in the cells grown on glass coverslips in the current experiments. While many experiments used cells on the edge of the monolayer (they load better with dye), cells in the middle of the monolayer also recovered from an acid load (at the predicted time) following addition of Na^+ perfusate (e.g. the cell of Fig. 6). No control has been made to evaluate either the tightness of the monolayer or accessibility of the basolateral membranes of LLC-PK_1 cells on glass coverslips. In addition, it is not known if a biased group of cells was selected for analysis on glass coverslips compared to the filterslip work, although the average resting pH_i of the cells is similar in both cases.

Much evidence supports the presence of a Na^+/H^+ exchange in the brush-border membrane of the proximal tubular cell which mediates Na^+ absorption and proton secretion (Murer et al., 1976; Aronson, 1981; Murer & Burkhardt, 1983; Sabolic & Burckhardt, 1983; Howlin et al., 1985). In addition, two studies were unable to detect Na^+/H^+ exchange in renal cortical basolateral membrane vesicles (Ives et al., 1983; Sabolic & Burckhardt, 1983). The results in LLC-PK_1 are therefore in apparent contradiction to an interpretation of the LLC-PK_1 cells as a model cell system for the proximal tubular epithelial cell. The homology of the LLC-PK_1 cell line to the proximal tubular epithelia is significant, but not absolute. The homology is based on the expression of Na^+/PO_4^- cotransport, Na^+/glucose cotransport, a variety of amino-acid transport systems, and other membranous and cytosolic enzymes (Rabito, 1981; Biber et al., 1983; Rabito & Karlish, 1983; Gstraunthaler et al., 1985). However, some hormonal receptors (for calcitonin and vasopressin) and levels of cytosolic enzymes in the LLC-PK_1 cells are more appropriate for distal sections of the renal tubule, as is the demonstration of Na^+, K^+ $2Cl^-$-cotransport (Dayer et al., 1981; Brown & Murer, 1985; Gstraunthaler et al., 1985). The basis for the expression of assorted renal functions may relate to adaptations to culture or cellular heterogeneity.

There are two broad categories which allow for an explanation of the current data. One is to assume that the cells are expressing the Na^+/H^+ exchanger in the wrong membrane. In this interpretation, either the intracellular signals have gone wrong (the route of membrane traffic is disturbed) and/or the extracellular signals are incorrect (inappropriate hormonal signals or extracellular matrix). Since the cells demonstrate normal polarized morphology, some trivial explanations requiring gross morphological changes may be excluded, but more subtle errors by the cells are possible, and the cells on filterslips were not evaluated for other membrane functions. It is known that the polarized morphology of cloned LLC-PK_1 cells (D+Sc) reverses when they are imbedded in collagen I gels (Wohlwend et al., 1985). This indicates that morphology is a parameter sensitive to changes in environment, and may therefore be a reasonable marker of the polarization state of some cellular proteins which are not directly visualized in the electron microscope.

The alternative broad explanation of the current data is that the cells are expressing the Na^+/H^+ exchanger in the correct membrane. In this interpretation, it is interesting that a polarized distribution of Na^+/H^+ exchange has been experimentally supported for the renal epithelial cells of both the proximal tubule and the cortical collecting tubule, but in opposing membranes (Ives et al., 1983; Sabolic & Burckhardt, 1983; Alpern et al., 1985; Chaillet et al., 1985; Sasaki et al., 1985; Yoshitomi et al., 1985). Thus, the LLC-PK_1 cells may be similar to the cortical collecting tubule cell which displays basolateral Na^+/H^+ exchange (Chaillet et al., 1985). It is also possible that the examined cells originated from the pig proximal tubule, but (under these culture conditions) are not expressing the Na^+/H^+ exchanger that is responsible for transepithelial transport, only an exchanger for pH_i regulation. The existence of multiple exchangers is possible and it has been demonstrated previously that the countercurrent distribution of Na^+/H^+ exchange is complex in a renal membrane vesicle population (Mircheff et al., 1984). Finally, it should be noted that the apical membrane of LLC-PK_1 cells contains relatively few microvilli compared to the proximal tubule cell (Nord et al., 1986). The ratio of membrane areas between the apical and basolateral membrane is therefore low compared to the proximal tubule cell. A polarized distribution of Na^+/H^+ exchange proteins in the proximal tubule cell may be due in part to the amplification of the apical membrane area by the microvilli. There are limits to this latter possibility because of the observed absence, in some LLC-PK_1 cells in this study, of any measurable pH_i recovery due to addition of Na^+ in the apical solutions. It is not possible currently to select between these possibilities, but using the capability to specifically evaluate the functional polarity of H^+ transport systems in tissue culture model systems, it is hoped that the answers may be attainable.

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