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Flow Cytometric Analysis of Intracellular pH in Cultured Opossum Kidney (OK) Cells

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Summary. Suspensions of OK cells (a continuous renal epithelial cell line originating from the opossum kidney) were examined by flow cytometry. Three parameters were evaluated simultaneously; cell integrity as assayed by propidium iodide fluorescence, cell size as measured by time-of-flight, and intracellular pH as measured by fluorescence of 2′,7′-*bis*-(2-carboxyethyl)-5,6 carboxyfluorescein (BCECF). The suspension was shown to be composed of both intact singlets and doublets of cells, and no difference was noted in the behavior of these two populations with respect to the resting intracellular pH, or of the response of intracellular BCECF to changes in pH. Evidence suggests that using NH_4 prepulses to create an acid load broadens the intracellular pH distribution. The population of OK cells demonstrates a recovery from this acid load which is very homogeneous with respect to its sensitivity to Na^+ removal or EIPA (ethylisopropyl-amiloride), suggesting that virtually all cells utilize Na^+/H^+ exchange for this recovery. The data also suggest heterogeneity in the cellular pH recovery from an acid load with respect to the observed rates of Na^+/H^+ exchange. Despite this heterogeneity, the Na^+/H^+ exchanger is observed to focus the resting intracellular pH of the population to approximately pH 7.4–7.5. The response of the population to PTH suggests that the majority of cells respond to the hormone, and that the total Na^+/H^+ exchange in individual cells is only partially inhibited even in the presence of saturating PTH concentrations.

Key Words intracellular pH · BCECF · propidium iodide · time-of-flight · epithelial cell culture · opossum kidney

Introduction

In both the native epithelial cells of the proximal tubule and OK cells (the latter an established renal epithelial cell line from opossum kidney), parathyroid hormone (PTH)[1] causes a decrease in the rate of Na^+/H^+ exchange and Na^+/phosphate cotransport in the plasma membrane (Kahn et al., 1985; Gmaj & Murer; 1986; Malmström & Murer, 1986; Pollock, Warnock & Strewler, 1986; Miller & Pollock, 1987; Moran, Montrose & Murer, 1988). In the native tissue, this is proposed as one mechanism whereby PTH decreases bicarbonate and phosphate reabsorption by the proximal tubule (Iino & Burg, 1979; Kahn et al., 1985; Gmaj & Murer, 1986). For these reasons, OK cells have been used previously as a model system with which to examine the cellular events leading to regulation of bicarbonate and phosphate reabsorption by the renal proximal tubule (Miller & Pollock, 1987; Malmström & Murer, 1987; Malmström, Stange & Murer, 1988; Moran et al., 1988).

In the response of both OK cells and native renal tissue to PTH, it has been noted that saturating concentrations of PTH lead to only an incomplete inhibition of Na^+/H^+ exchange (50–60% inhibition) (Kahn et al., 1985; Pollock et al., 1986; Miller & Pollock, 1987; Moran et al., 1988). It is not known whether the basis for the incomplete inhibition of the Na^+/H^+ exchange is due to heterogeneity in the response to PTH between different cells, or whether it is due to other factors. This is only one of a number of questions that relate to the homogeneity of cell population responses. Since the rate of Na^+/H^+ exchange is usually measured under conditions of an acid load (a condition that activates Na^+/H^+ exchange), it may also be important to evaluate whether the response to cellular acidification and the basal rate of transport have any intrinsic heterogeneity.

Flow cytometry has been shown to be useful in gathering information from single cells while still retaining information at the level of whole populations. Because flow cytometry allows an analysis of variability with respect to any measured parameter in a population, the technique can serve to bridge the gap between microfluorometric studies on indi-

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[1] *Abbreviations used:* BCECF, 2′,7′-*bis*-(2-carboxyethyl)-5,6 carboxyfluorescein; CV, coefficient of variation; EIPA, 5-(N-ethyl)-N-isopropyl-amiloride: HEPES, 4-(-2-hydroxyethyl)-1-piperazine ethansulfonic acid; MES, 2[N-morpholino] ethansulfonic acid; pH_i, cytoplasmic pH; PI, propidium iodide; PTH, parathyroid hormone; TMA^+, tetramethylammonium.

vidual cells (Montrose et al., 1987) and measurements made on (many) suspended cells in a standard fluorometer cuvette (Moran et al., 1988). Flow cytometric techniques have been established previously in other cells for measurement of intracellular pH (pH_i) using dyes with pH-sensitive emission spectra (Valet, Raffael & Moroder, 1981; Gerson, 1982), as well as procedures for the analysis of cell size and cell integrity (Shapiro, 1986). To our knowledge, only two reports utilize BCECF (a fluorescent dye with a pH-sensitive spectrum) for flow cytometric measurement of intracellular pH (Cantiello, Scott & Rabito, 1986; Musgrove, Rugg & Hedley, 1986), despite the desirable compatibility of its spectrum with both a known viability dye (propidium iodide; PI) (Shapiro, 1986), and the 488 line of the argon ion laser. Given the information already available concerning the presence of PTH-sensitive Na^+/H^+ exchange in suspended OK cells (Montrose et al., 1988; Moran et al., 1988), flow cytometry is an appropriate tool with which to examine questions about the homogeneity or heterogeneity of OK cell responses.

The goal of these studies is to collect flow cytometric information concerning the population of suspended OK cells with respect to the parameters of cell size, cell integrity, and cell pH. In addition to establishing conditions for use and analysis of OK cells by flow cytometry, experiments have been performed to determine whether the population of OK cells is homogeneous or heterogeneous with respect to cellular acidification, cellular recovery from an acid load, and the hormonal response to parathyroid hormone.

Materials and Methods

Cell Culture and Cell Suspension

OK cells (Koyama et al., 1978) were originally obtained from Dr. D.G. Warnock (San Francisco, CA) at serial passage 79, and experiments were performed using cells between passage 86 and 96. Cells were maintained in culture in a humidified 5% CO_2 (95% air) atmosphere and were subcultured as previously described (Malmström et al., 1988). For experimental purposes, 3×10^6 cells were seeded onto 75 cm^2 culture flasks (Corning, New York) and grown (to confluency) for 4–6 days. Cell suspensions were created by a 3–5 min incubation of the cell monolayer (at 37°C) in 2 ml of Ca^{2+}-Mg^{2+}-free phosphate buffered saline containing 0.1% trypsin (Flow Laboratories, Scotland) plus 0.5 mM EDTA. The cells were then diluted into 20 ml of medium (described below) containing 6 mg of ovomucoid trypsin inhibitor (T-9253, Sigma, St. Louis, MO) and left for at least 4 hr at 25°C to reequilibrate while being gently shaken. After this interval, aliquots of the material were used at 25°C for all ensuing work with cell suspensions.

Solutions

Cells were suspended in a medium resembling culture medium in its inorganic ionic content and osmolality (denoted *Na^+ medium*, and containing in mM: 145 Na^+, 4 K^+, 1 Ca^{2+}, 1 Mg^{2+}, 136 Cl^-, 1 SO_4^{2-}, 1 phosphate, 20 HEPES, 18 glucose, pH 7.4 at 25°C). For some experiments, cells were rapidly resuspended in a medium in which Na^+ was replaced with TMA^+ (*TMA^+ medium*). In other experiments, cells were placed in *high K^+ medium* containing in mM: 110 K^+, 17 Na^+, 25 TMA^+, 1 Ca^{2+}, 1 Mg^{2+}, 102 gluconate, 25 Cl^-, 1 SO_4^{2-}, 1 PO_4^-, 18 glucose, 15 MES, 15 HEPES, pH 7.4. In other work, this concentration of K^+ was determined (by flame photometry) to approximate intracellular K^+ concentration (M.H. Montrose and H. Murer, *in preparation*).

Use of Cells for Flow Cytometry

Routinely, cells to be examined in the flow cytometer were loaded with BCECF (a fluorescent dye with a pH-sensitive spectrum) for evaluation of intracellular pH. The dye loading was performed by exposure of 2 ml of the cell suspension to 1 μM of the acetoxymethyl ester of BCECF (Molecular Probes, OR) at room temperature in Na^+ medium for 45–60 min. If cells were to be exposed to (30 mM) NH_4Cl and/or (10^{-7} M) parathyroid hormone, the agents were added in the last 15 min of the dye-loading interval. Dye-loaded cells were centrifuged (1 sec) in an Eppendorf 5412 centrifuge, the supernatant was aspirated, and the cell pellet quickly resuspended in 50 μl of the desired experimental medium. Cells were then injected into 2 ml of the experimental medium containing 5 μg/ml propidium iodide (Molecular Probes) and stirred with a small Teflon stir bar. The passage of cells through the flow chamber of the cytometer was then started as quickly as possible (data collection was routinely started 15–20 sec after resuspension of the cell pellet). The flow of cells through the flow chamber was maintained at roughly 200 cells per second and was monitored (and adjusted if necessary) throughout an experiment.

Flow Cytometry

A Cytofluorograf 50H coupled to the 2151 computer system was used in these studies (Ortho Diagnostics, MA). The laser was a Spectra Physics 164-05 argon ion laser running at 100 mW with the 488 nm line. The 90° light leaving the flow chamber was directed through a 510 long pass filter, and then a 585 short pass filter was used as a dichroic reflecting mirror. The shorter (passed) wavelengths were then directed through a 510–555 nm bandpass filter for measurement of green (BCECF) fluorescence. The longer (reflected) wavelengths were directed through a 630 nm long pass filter for collection of red (propidium iodide; PI) fluorescence. Routinely, the strong green fluorescence was observed to spill over into the red channel and required compensation. This compensation was performed by subtracting a fixed percentage of the green fluorescence value from the red fluorescence channel for each measured cell. This fixed percentage was 30–60% in different experiments and was set such that a residual small spillover was still detectable (*see* Fig. 1*A*). For each preparation, digitonin-permeabilized cells (*see* Fig. 1*B*) were used to correctly set the value of compensation and to identify the value of red fluorescence which corresponded to cells positively stained with PI.

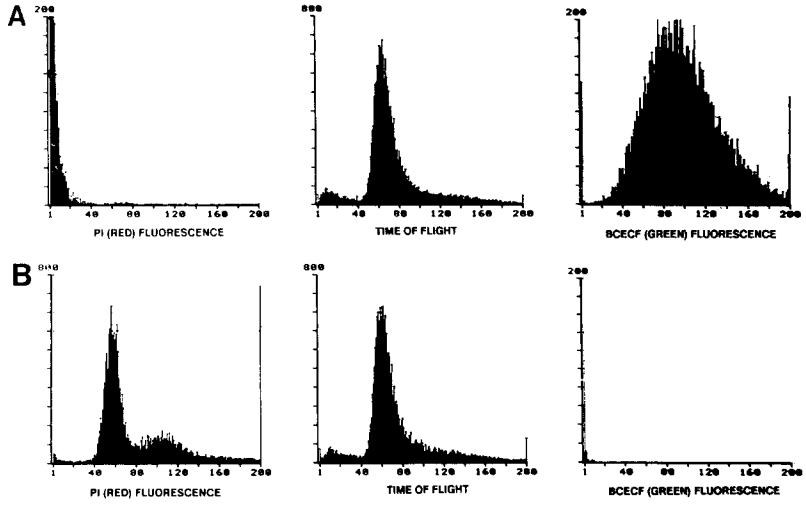

Fig. 1. Comparison of propidium iodide, time-of-flight, and BCECF measurements before and after digitonin addition. All histograms present the number of cells on the vertical axis versus the magnitude of the parameter indicated on the horizontal axis. (*A*) Results of data collection of 20,000 OK cells loaded with BCECF and exposed to Na^+ medium containing 5 μg/ml propidium iodide (PI). A comparison is presented of the three independent parameters as they are measured for each cell as it passes through the laser beam. (*B*) Results of data collection of 20,000 cells from the same cell suspension 1 min after addition of 50 μM digitonin. Similar results were seen in five preparations

Low-angle light scatter was used to gate for the presence of cells and was also used to determine the time-of-flight of cells passing through the (5 μm diameter) laser beam. Time-of-flight was evaluated under conditions of maximum sensitivity on the detector and zero offset on the time-of-flight amplifier. Time-of-flight was determined to be proportional to diameter when flowing latex beads of 10, 17, and 25 μm (Coulter and Polysciences) through the laser beam (*data not shown*). The mean diameter of single OK cells was 13–16 μm in these experiments.

Miscellaneous Techniques

Nigericin (Calbiochem) was added to solutions from a 10 mM stock in ethanol, propidium iodide (Molecular Probes) from a 5 mg/ml solution in water, and digitonin (Calbiochem) from a 20 mM solution in DMSO. Parathyroid hormone was purchased from Bachem, Switzerland (bovine 1-34 synthetic parathyroid hormone; PTH). All averaged data are presented as mean ± SEM.

Results

It was first evaluated whether it was possible to measure cell integrity, cell size, and cell pH simultaneously in the flow cytometer in a reasonable manner. To measure cell integrity, we have used the (red fluorescent) dye propidium iodide (PI), which stains double-stranded nucleic acids (Crissman & Steinkamp, 1973; Frankfurt, 1980; Shapiro, 1986). This compound is normally impermeant to cells (and cells remain unstained) as long as membrane integrity (cell viability) is intact. To measure cell size, we have used the time-of-flight of cells as they pass through the flow cytometer laser beam as a direct measure of cell diameter. To measure intracellular pH, we have used BCECF (a fluorescent dye with a pH-sensitive spectrum). Figure 1*A* presents the information collected from a total of 20,000 cells, where all these parameters are measured simultaneously from individual cells as they pass through the laser beam.[2] As shown in Fig. 1*A*, the population of OK cells has very few cells with PI fluorescence above 40 units, although a significant amount of fluorescence of low intensity is detected in the red channel. Work with cells loaded with BCECF, but without PI in the medium, indicates that this low intensity fluorescence is due to the strong BCECF fluorescence spilling over into the red channel (*data not shown*). Figure 1*A* also demonstrates a major peak of time-of-flight. In most preparations, a more distinct second (minor) peak was observed at time-of-flight values higher than the major peak. This suggests that both singlets (major peak) and doublets of cells may be present in the suspended population. Finally, Fig. 1*A* demonstrates the single peak of BCECF fluorescence in the unperturbed cell population.

In contrast to these values observed in 20,000 unperturbed OK cells, 20,000 cells were also analyzed approximately 1 min after addition of 50 μM digitonin. In this population, a major and minor peak of PI appears, the time-of-flight measurement is minimally changed, and the BCECF peak is completely gone. This is all shown in Fig. 1*B*. Note that in the case of PI, the low-intensity fluorescence is also gone, as predicted if the values are due to BCECF fluorescence spillover. It was always possible to clearly distinguish between cells that were truly stained with PI (i.e., fluorescence above 40 units in Fig. 1), and the crossover from the BCECF

[2] Please note that all the graphs presented in the first four figures are histograms of cell number versus the magnitude of the measured parameter.

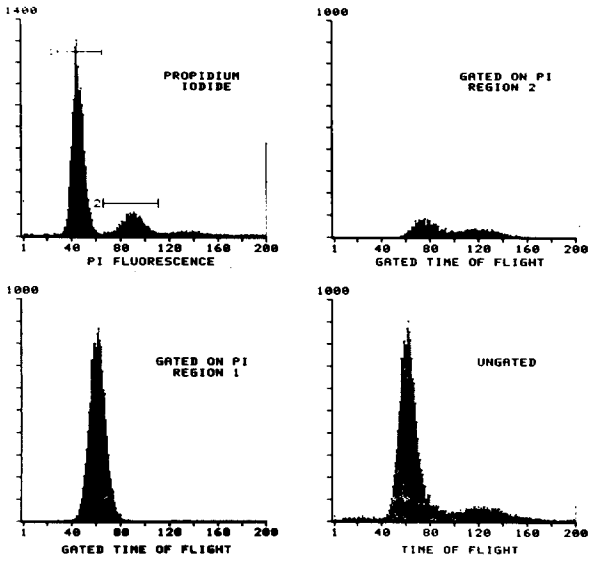

Fig. 2. Gating of the time-of-flight distribution by propidium iodide fluorescence. Cell suspensions that had been treated with 50 μM digitonin were examined. Cell number is presented on the vertical axis of all histograms. Regions of the propidium iodide (PI) distribution are selected (regions 1 and 2 of the upper left histogram) and the time-of-flight of the cells within these regions are presented in the lower left and upper right histograms. The original (ungated) time-of-flight distribution is presented in the lower right histogram for comparison. Similar results were seen in three preparations

fluorescence. The experiment of Fig. 1 suggests that PI may be a useful tool in discriminating live from dead cells. In the experiments reported in this paper, the percent of the cell population stained with PI was typically 0.2–0.7% before permeabilization and greater than 95% after permeabilization (*data not shown*). Since the time-of-flight measurement is minimally changed after cell permeabilization, the experiment also implies that cell size and cell detection are not strongly affected by the digitonin treatment. Finally, the complete loss of BCECF fluorescence signal after digitonin treatment (due to loss of BCECF from the cells implies that any extracellular BCECF present does not contribute to the measurement of intracellular BCECF.

Since the data of Fig. 1 suggest that it is possible to stain cells with PI and not change the time-of-flight signal, it is possible to analyze the singlet and doublet peaks in more detail to see if the peaks logically correlate with singles and doubles of cells passing through the laser beam. For this purpose, we have treated cells with 50 μM digitonin, collected (list mode) data from a large number of cells, and then after the experiment reanalyzed the data to examine in more detail subgroups of the entire population. We first wanted to analyze whether cells which were contained in the separate peaks of PI fluorescence correlated with peaks in the time-of-flight. As shown in the upper left histogram of Fig. 2, it is possible to select regions of the PI fluorescence and determine the time-of-flight values for the cells collected within these gated regions. In contrast to the entire (ungated) distribution of time-of-flight shown in the lower right histogram of Fig. 2, the two gated time-of-flight distributions are significantly different. The lower left histogram suggests that the only cells with a single copy of DNA (region 1 of the PI histogram) are those cells within the major time-of-flight peak. No doublets in time-of-flight were observed to have a single copy of DNA. In contrast, the upper right histogram suggests that those cells with double the amount of DNA (region 2 of the PI histogram) have a more complex time-of-flight distribution. These cells include (as predicted) the doublets in the time-of-flight, but also include a smaller peak which is nonetheless larger than the major (singlet) peak (1.26 ± 0.002-fold higher in three experiments). The latter cells are likely to be cells in G2 and late S phases (cells that have increased nucleic acid content and size, but have not divided). The results of Fig. 2 indicate that the major and minor time-of-flight peaks are likely to represent single and double cells, respectively. The chance of coincidence of two unattached cells in the laser beam is very low with the slow rates of cell analysis used here. Therefore, the doublets of cells are probably due to cells that were not separated during the trypsinization leading to cell suspension. In four preparations, the single cells were found to comprise 76 ± 4% of the total cell population.

Given that the time-of-flight peaks indicate both single and double cells in the population, we desired to use the time-of-flight measurement routinely to allow us to analyze selectively either singlets or doublets of cells in the population. First, it is possible to perform a related analysis on the data contained in Fig. 2, to characterize the subgroups of cells in the time-of-flight histogram with respect to PI fluorescence. As shown in the upper left histogram of Fig. 3, regions corresponding to singlets and doublets of (time-of-flight) cells may be selected to examine how much nucleic acid they contain. In contrast to the ungated histogram of PI fluorescence shown at the lower right, the lower left histogram indicates that the time-of-flight singlets (region 1 of the time-of-flight histogram) have fewer cells with doublets of PI fluorescence. The percentage of single cells with doublets of DNA (14 ± 3% in three preparations) may give an indication of the amount of cells in late S and G2 phase. As predicted, time-of-flight doublets (region 2 of the time-of-flight his-

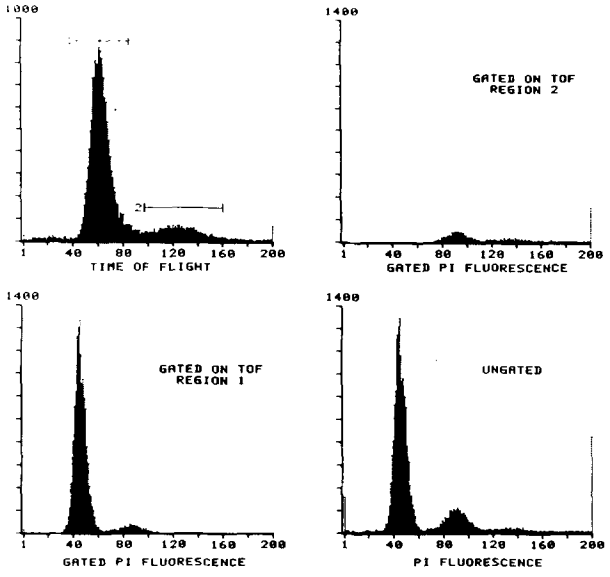

Fig. 3. Gating of the propidium iodide fluorescence by time-of-flight. Cell suspensions that had been treated with 50 μM digitonin were examined. Cell number is presented on the vertical axis of all histograms. Regions of the time-of-flight (TOF) distribution are selected (regions 1 and 2 of the upper left histogram) and the propidium iodide fluorescence of the cells within these regions are presented in the lower left and upper right histograms. The original (ungated) propidium iodide fluorescence distribution is presented in the lower right histogram for comparison. Similar results were observed in three preparations

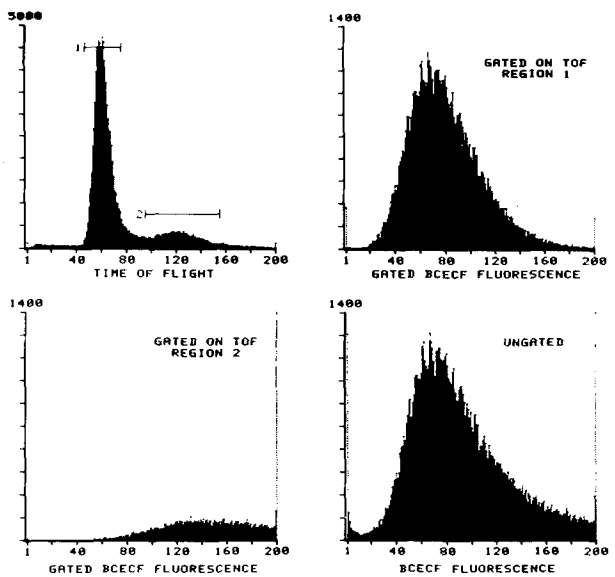

Fig. 4. Gating of BCECF fluorescence by the time-of-flight signal. Cells suspended in Na^+ medium were examined. Cell number is presented on the vertical axis of all histograms. Regions of the time-of-flight (TOF) distribution are selected (regions 1 and 2 of the upper left histogram) and the BCECF fluorescence of the cells within these regions are presented in the lower left and upper right histograms. The original (ungated) BCECF fluorescence distribution is presented in the lower right histogram for comparison. Similar results were observed in four preparations

togram) never contain only one copy of DNA (as shown in the upper right histogram of Fig. 3).

We next wanted to evaluate the effect of time-of-flight gating on the BCECF signal. As shown in Fig. 4, by gating on time-of-flight singlets (region 1 of the upper left histogram), it is possible to convert the histogram of BCECF fluorescence (ungated in the lower right histogram) to a more symmetrical distribution (upper right histogram) which is fully contained within the measurable range of fluorescence intensities (i.e., no data is "off-scale"). Both of these facts are helpful when attempting to characterize the population distribution in terms of mean fluorescence and the variation within the observed values. Figure 4 also depicts the effect on BCECF fluorescence of gating on the doublets of cells (gating on region 2 of the time-of-flight histogram). As shown (lower left histogram), doublets of cells were observed to have significantly higher fluorescence. We have also performed BCECF gating on time-of-flight. Both halves of the BCECF distribution above and below the peak channel (bright and dim cells, respectively) contain a singlet time-of-flight peak. However, the brighter cells have a singlet peak whose mean time-of-flight was 115 ± 4% (n = 3 preparations, mean ± SD) of the singlet peak of dim cells. Thus part of the reason for a broad BCECF distribution may be that slightly larger cells load more dye.

It was observed in four preparations that the time-of-flight doublet peak had a mean time-of-flight value which was 1.95 ± 0.02-fold higher than the time-of-flight singlet peak. Similarly, the mean fluorescence of the PI doublet peak was 1.98 ± 0.02-fold higher than the PI singlet peak. This suggests that in both cases the measurement is extremely close to the value predicted if the measurements relate to singlets versus doublets of cells (i.e., two). In contrast, in the same four preparations the BCECF fluorescence of the (time-of-flight) doublets was only 1.70 ± 0.03-fold higher than the (time-of-flight) singlets when examining the fluorescence at resting pH_i. This is not likely to relate to differences in pH_i between singlets and doublets, because in cells pH clamped with high K^+ medium and nigericin (at a mean BCECF fluorescence which was 89% of the resting pH_i value), the doublets were still only 1.75 ± 0.09-fold higher than the singlets. The data suggest that the doublets are not loading with BCECF as well as the singlets in the population, or that they have lost BCECF more quickly after loading. In all further discussion, "singlets" and "dou-

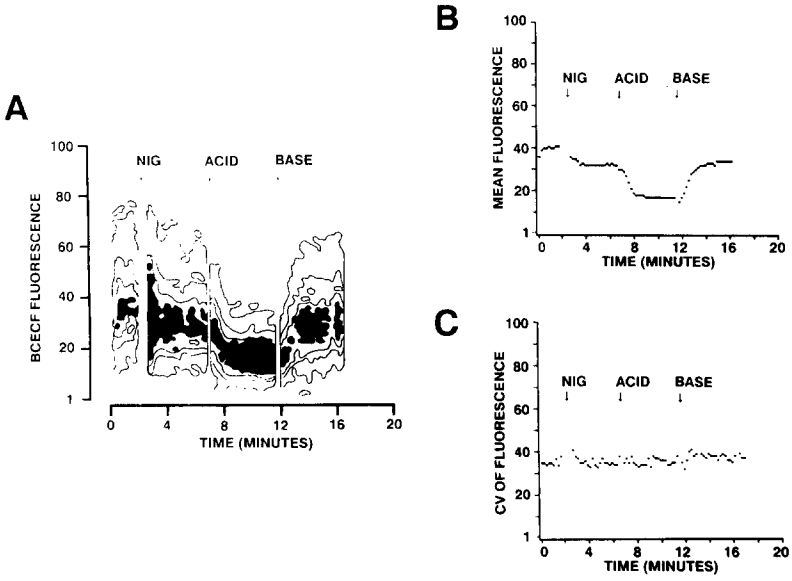

Fig. 5. Analysis of cells pH clamped with high K^+ medium and nigericin. Cells were rapidly resuspended in high K^+ medium and examined in the flow cytometer. After data collection, a double gating was performed. The cells depicted were gated for low PI fluorescence (viable cells), and to be within the major peak of time-of-flight (single cells). Final concentrations of 10 μM nigericin (*NIG*), 5 mM HNO_3 (*ACID*), and 5 mM TMA-OH (*BASE*) were added to the suspension at the times indicated by the arrows. (*A*) A contour plot of BCECF fluorescence versus time. The figure is a 2-dimensional representation of 3-dimensions of data where the *z* axis is cell number. The most inclusive contour boundary defines the recording of a single cell and, with equal increments of cell number, subsequent lines present boundaries defining higher numbers of observed cells. (*B*) The data shown in Fig. 1*A* is analyzed such that for every 12-sec interval, the mean of the collected population is calculated. The calculated mean is presented versus time. (*C*) The data shown in Fig. 1*A* is analyzed such that for every 12-sec interval, the coefficient of variation (CV) is calculated. The calculated CV is presented versus time

blets" will be assumed to refer to the cell populations as defined by time-of-flight measurements.

In order to demonstrate that the flow cytometer could detect changes in BCECF fluorescence when cell pH was changed, cells were rapidly resuspended in high K^+ medium and analyzed in the flow cytometer in the absence and presence of nigericin (a K^+/H^+ exchanger). The data shown in Fig. 5 were collected such that the computer stored data collected over a fixed time interval (12 sec) and then immediately started the next time interval. At the end of an experiment, data were double-gated so that only intact (i.e., low PI fluorescence), single (i.e., the major time-of-flight peak) cells would be analyzed.[3] Figure 5*A* shows a contour plot of the resultant BCECF fluorescence (the contour lines represent equal number of cells). As shown, acidification of the medium to pH 6.6 (in the presence of 10 μM nigericin) caused a decrease in the fluorescence of the entire population of single cells. Subsequent alkalinization to pH 7.5 caused a return of higher fluorescence levels. These data are analyzed more quantitatively in Fig. 5*B* and *C*. In Fig. 5*B*, the mean fluorescence calculated for each 12-sec interval from Fig. 5*A* is presented. Essentially this is a reconstruction (with poor time resolution) of what would be observed in a normal fluorometer cuvette, if one could selectively analyze the single cells in the population. Similar results were seen (with higher average fluorescence values) when doublets of cells were analyzed (*data not shown*).

In Fig. 5*C*, the variability of the population is evaluated using the coefficient of variation (CV). The CV is the standard deviation (SD) of the population, normalized to be a percentage of the population mean (i.e., CV = [SD/mean] × 100). Note that upon addition of nigericin, the steady-state CV does not change. This implies that the imposition of a pH clamp does not produce any noticeable "sharpening" of the fluorescence distribution. That is, the cellular pH of suspended OK cells is as homogeneous before as after pH-clamp imposition. This was observed in five separate analyses using both

[3] During 20 min of data collection, a slight but significant increase in the amount of PI-positive cells was observed. However, this amount was never greater than 1% of the cells, and, with the exception of digitonin addition or an increase in medium pH above pH 9.5, no other treatment or addition was observed to affect PI fluorescence.

singlets and doublets of cells (CV post-nigericin was 101 ± 5% of the CV pre-nigericin). It is known that the high K^+ medium itself did not affect the distribution, since the value of mean fluorescence and CV were the same for cells in high K^+ medium without nigericin (40 units and 35%, respectively) as for cells in Na^+ medium (39 units and 36%, respectively). As also shown in Fig. 5*C*, over the range of fluorescence values encountered in the pH-clamped cells, the CV does not change. The simplest explanation of these latter data are (i) that the entire population of single cells is changing fluorescence proportionally (as suggested qualitatively by Fig. 5*A*), and (ii) that the CV is a function of the variability of the cell population, such that the variability due to the flow cytometer electronics is negligible under these conditions.

The experimental protocol presented in Fig. 5 may be used to calibrate the dye response of cell singlets independently from cell doublets. Results from four preparations analyzed in this manner are presented in Fig. 6. When the mean BCECF fluorescence of both singlets and doublets is normalized to a value of 100 for cells pH clamped at pH 7.3 (with high K^+ medium and nigericin), subsequent changes in medium pH produce similar shifts in mean (normalized) fluorescence for both populations. The observed pH sensitivity of singlets and doublets may also be compared to the pH sensitivity of unesterified BCECF in solution. The line in Fig. 6 shows the pH sensitivity of (50 nM) BCECF fluorescence in a standard fluorometer cuvette when the fluorometer is used with excitation and emission settings similar to those of the flow cytometer. The results in Fig. 6 imply that in terms of the pH sensitivity of intracellular BCECF, both singlets and doublets of cells behave similarly, and as expected based on results using unesterified BCECF. The resting pH_i of the singlets (7.48 ± 0.14) versus the doublets (7.46 ± 0.07) in the population may also be compared using the calibration curve shown in the figure. No difference in the resting pH_i was detected between these two populations of OK cells.

As shown in Fig. 7, in unperturbed cells the BCECF fluorescence is virtually constant over long periods of time. This is evident whether one evaluates the population by looking at a contour plot of the cell distribution (*A*), the mean fluorescence (*B*), or the CV of the distribution (*C*). This strongly suggests that the loss of BCECF from the cells must be extremely slow under these conditions.

If OK cells are exposed to 30 mM NH_4Cl during the last 15 min of dye loading with BCECF, a subsequent removal of the NH_4 leads to an intracellular acid load (Moran et al., 1988; Montrose & Murer (*in preparation*). The response of the single, intact cells to this acid load is shown in Fig. 8, with data collection starting 20 sec after removal of NH_4, and with suspended cells in Na^+ medium. As shown in Fig. 8*B*, the mean BCECF fluorescence of the cells is significantly decreased at early time points (cellular acidification), but increases with time (pH_i recovery). In unperturbed single, intact cells from the same preparation, the resting mean fluorescence was 35 units and the CV was 40%. As shown in Fig. 8*C*, the CV indicates that during the early phases of cellular acidification and pH_i recovery the distribution transiently broadens (CV has increased) before returning to the original value of the resting cells. Note that after the first minute of data collection, the increase in CV is no longer correlated with a decrease in BCECF mean fluorescence. This implies that the broadening is not simply a function of instrument noise, but must be due to an increased variability in cellular pH. The data suggest that the

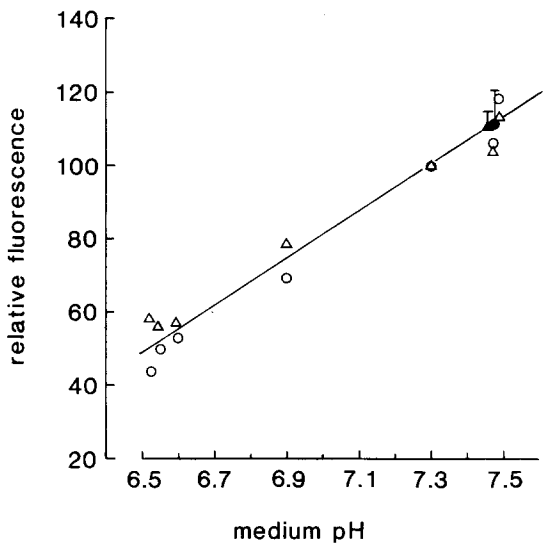

Fig. 6. Calibration curves of single cells are compared to doublets of cells. Data such as that of Fig. 5*B* is presented for both time-of-flight singlets (○,●) and time-of-flight doublets (△,▲) of cells. Cells are compared in the absence (●,▲) and presence (○,△) of 10 μM nigericin. All data are normalized to a value of 100 fluorescent units for the fluorescence observed in cells treated with nigericin and suspended in high K^+ medium of pH 7.3. The line in the figure is the response of 50 nM BCECF in solution (also normalized to a value of 100 units at pH 7.3). The solution of 50 nM BCECF was analyzed in a standard fluorometer (Shimadzu RF-510) with excitation of 488 nm (3 nm bandwidth) and emission of 530 nm (40 nm bandwidth). The data in the presence of nigericin are single analyses of a population and are collected from four preparations. The data in the absence of nigericin are the mean ± SEM of the analyses of the same four preparations, with the relative fluorescence values placed at the appropriate location on the calibration line

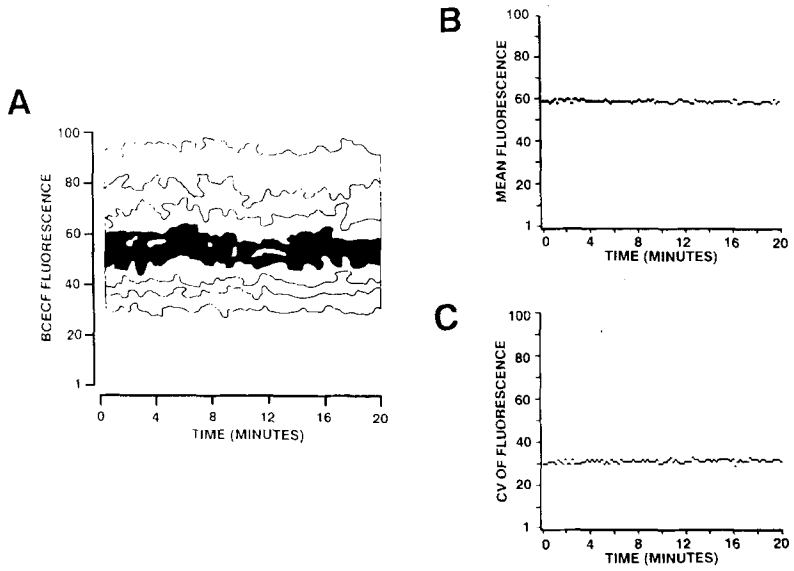

Fig. 7. Analysis of the steady-state pH_i (BCECF fluorescence) of unperturbed cells in Na^+ medium. All cells in the preparation are presented (i.e., no gating of the results was made). Otherwise, the data were analyzed exactly as described in Fig. 6. The amount of PI-stained cells collected during the experiment was 0.7% of the entire population. Similar results were observed in three preparations

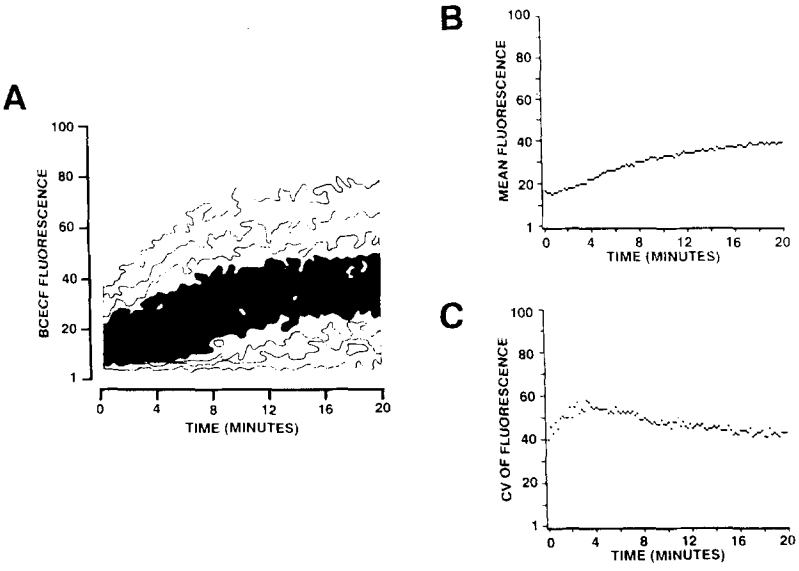

Fig. 8. Analysis of the cellular pH_i recovery from an acid load, when cells are resuspended in Na^+ medium. The acid load was imposed by incubation with 30 mM NH_4Cl during the last 15-min interval of dye loading. The subsequent centrifugation and resuspension which is used to remove extracellular dye was also used to remove NH_4 from the medium. The results were double gated, so that the results from single, viable cells are presented and analyzed exactly as described in Fig. 6. Similar results were observed in five preparations

cellular response to the NH_4-prepulse acid load is heterogeneous and/or that the cellular recovery from the acid load is heterogeneous. By looking at Fig. 8*A*, it is possible to see that any heterogeneity in cellular response would have to be due either to a population of cells that recovers more quickly than average, or to cells that are less acidified than average. It is interesting to note that whatever the cause of the pH variability, the cellular recovery from an acid load acts to remove it. This "focussing" of intracellular pH_i was observed in five preparations and is strong evidence that the transport system catalyzing the recovery has a rate modulated by pH_i.

In Fig. 9, the response of (both single and double) live cells to an (NH_4 prepulse) acid load is evaluated in medium with TMA^+ replacing Na^+. As shown in Fig. 9*B*, in comparison to the pH_i recovery observed in Na^+ medium (in the same preparation with the same method of analysis), substitution of TMA^+ medium strongly blunts the recovery. Figure 9*A* indicates that there is no identifiable subpopulation of cells able to escape quickly from the cellular acidification in Na^+-free medium. In unperturbed cells, the mean BCECF fluorescence was 59 units and the CV was 31%. By the end of the data collection interval, the CV of cells in Na^+ medium had decreased to 36 from a maximum of 57%.

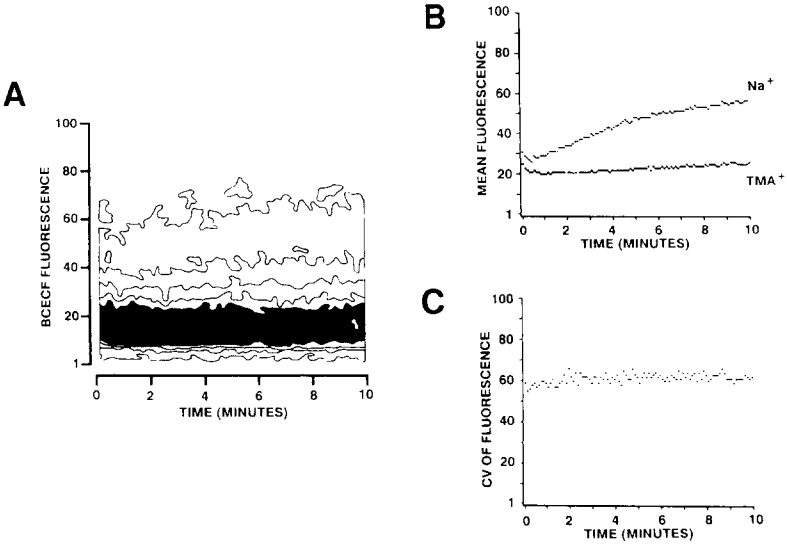

Fig. 9. Analysis of the cellular pH_i recovery from an acid load, when cells are resuspended in TMA^+ medium. The acid load was imposed by incubation with 30 mM NH_4Cl during the last 15-min interval of dye loading. The subsequent centrifugation and resuspension which is used to remove extracellular dye was also used to remove NH_4 and Na^+ from the medium. In this experiment, the results are from both singlets and doublets of viable cells, and the analysis time interval was 6 (and not 12) sec; otherwise, data analysis is as described in Fig. 6(*B*). This figure includes a second run for comparison (from the same preparation and analyzed in the same way) using cells resuspended in Na^+ medium. Similar results were observed in four preparations

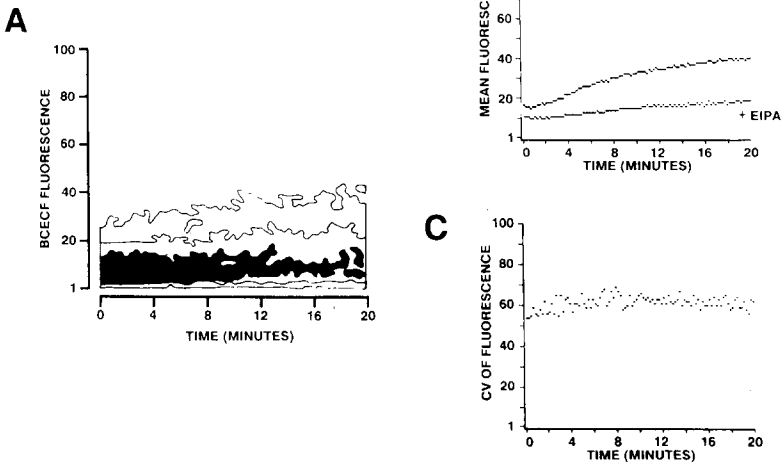

Fig. 10. Analysis of the cellular pH_i recovery from an acid load, when cells are resuspended in Na^+ medium plus 20 μM EIPA. The acid load was imposed by incubation with 30 mM NH_4Cl during the last 15-min interval of dye loading. Cells were exposed to EIPA 15 sec prior to the start of data collection. The results were double gated, so that the results from single, viable cells are presented and analyzed exactly as described in Fig. 6(*B*). This figure includes a second run for comparison (from the same preparation and analyzed in the same way) using cells resuspended in Na^+ medium without EIPA. Similar results were observed in four preparations

As shown in Fig. 9*C*, the data indicates clearly that the focussing of pH_i requires a pH_i recovery, since cells in TMA^+ medium do not demonstrate pH_i focussing (i.e., the CV remained high). Also note that the increase in CV occurs under conditions where negligible pH_i recovery takes place. This suggests that heterogeneity of acidification is most likely to explain the majority of the observed heterogeneity of pH_i.

In Fig. 10, the response of single, live cells to an (NH_4 prepulse) acid load is evaluated in medium with 20 μM EIPA (5-(N-ethyl)-N-isopropyl-amiloride) added to Na^+ medium at time zero. As shown in Fig. 10*B*, the pH_i recovery is strongly blunted compared to the absence of EIPA. Note that the same cell population (in normal Na^+ medium) has been analyzed in detail in Fig. 8. As shown qualitatively in Fig. 10*A*, no large subpopulation of the OK cells is able to mount a fast recovery in the presence of 20 μM EIPA. However, it is evident from Fig. 10*C* that the final increase in CV is observed to reach a maximum with a slower time course than that observed previously with faster pH_i recovery (*see* Fig. 8). This was noted in five determinations from three preparations, in which the maximal CV in the presence of EIPA took almost twice as long to be attained (1.97 ± 0.11-fold longer; $n = 5$) as in the absence of EIPA. This suggests that, in addition to the pH_i broadening due to a heterogeneity of acidification, a slight hetereoge-

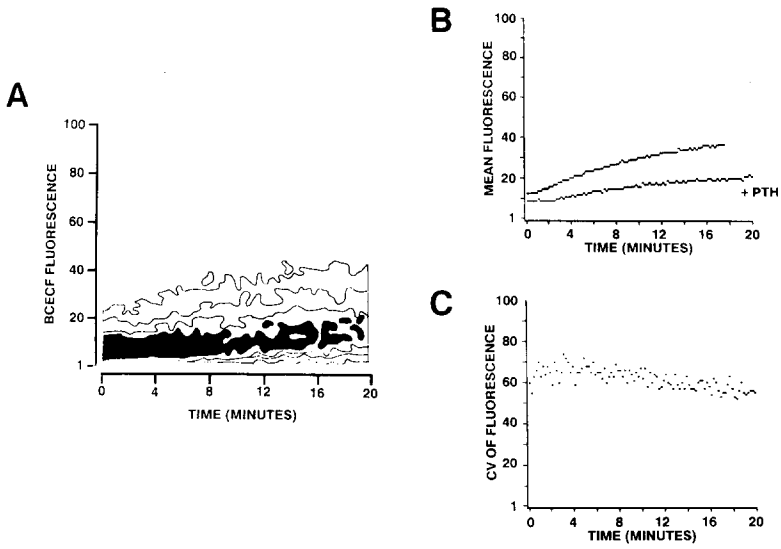

Fig. 11. Analysis of the cellular pH_i recovery from an acid load, when cells are exposed to 100 nm PTH and resuspended in Na^+ medium. Cells were exposed to 30 mM NH_4Cl and 100 nM PTH during the last 15-min interval of dye loading, and 100 nM PTH was included in the final resuspension medium. The results were double gated, so that the results from single, viable cells are presented and analyzed exactly as described in Fig. 6(*B*). This figure includes a second run for comparison (from the same preparation and analyzed in the same way) using cells which were not exposed to PTH. Similar results were observed in four preparations

neity is also generated during the pH_i recovery. This suggests slightly different rates of pH_i recovery are observed between individual cells. The data of Figs. 9 and 10 suggest that Na^+/H^+ exchange (a Na^+-dependent and EIPA-sensitive process) is responsible for the recovery from an acid load in the vast majority of OK cells.

Since Na^+/H^+ exchange has been shown to be decreased in OK cells in response to PTH (Pollock et al., 1986; Miller & Pollock, 1987; Moran et al., 1988; Montrose et al., 1988), we desired to test whether cells in the population responded equally to PTH. For this purpose we have evaluated the response of single, intact cells in Na^+ medium to an (NH_4 prepulse) acid load, after pre-exposure to 10^{-7} M PTH for 15 min. As shown in Fig. 11*A* and *C*, the pH_i recovery in the presence of PTH does not demonstrate evidence of a strong heterogeneity in the population, other than that which has been noted earlier due to other factors. The data may be compared to the control run shown for comparison in Fig. 11*B*. In the absence of PTH, the CV was observed to reach a maximum of 60% at 3 min, and decreased to 42% by 17 min. The data suggest that the majority of OK cells respond to PTH by lowering their rate of transport by the same fractional amount.

Discussion

OK cells are currently one of the best cellular model systems for studying some of the functions of the renal proximal tubule. These cells express a variety of transport systems appropriate for the epithelial cells of the proximal tubule (Malmström, Stange & Murer, 1987), as well as expressing PTH receptors and regulation of Na^+/phosphate cotransport and Na^+/H^+ exchange via interaction of PTH with these receptors (Malmström & Murer, 1986; Pollock et al., 1986; Miller & Pollock, 1987; Moran et al., 1988; Montrose et al., 1988). The latter observations suggest that the OK cells may be a suitable model for examining the effect of PTH to decrease both phosphate and bicarbonate reabsorption in the proximal tubule (Iino & Burg, 1979; Kahn et al., 1985; Gmaj & Murer, 1986).

The aim of this study was to initialize flow cytometric studies of OK cell suspensions, in order to analyze events at a single cell level while still accumulating information at the level of the entire population. In particular, we wished to characterize the cell population with respect to the homogeneity or heterogeneity of the cellular response to a variety of treatments that had previously been applied either using single cells, or within a standard fluorometer that averages many cells.

In order to use the flow cytometer, we first evaluated whether BCECF (a fluorescent dye with a pH-sensitive excitation spectrum), propidium iodide (PI; a nucleic acid dye which has been used previously in cytometric analyses of cell integrity and cell cycle) (Crissman & Steinkamp, 1973; Shapiro, 1986), and time-of-flight (a measurement of cell diameter) were appropriate tools with which to analyze the population. Digitonin permeabilization of the cells demonstrated that extracellular BCECF did not contribute to the green fluorescence signal, and that cells positively stained with PI could be clearly identified. Cross correlations of PI fluores-

cence and time-of-flight were useful in evaluating that the peaks observed in the time-of-flight and PI measurements related to singlets and doublets of cells. It is worth noting that under the conditions used (no added RNAase), the propidium iodide is probably not specific for DNA, but also senses double-stranded RNA (Frankfurt, 1980). This explains the relatively large CV's which are observed in the OK cell PI distributions (typically 10%) compared to more rigorous DNA analyses (typically 3–5%). However, the peak broadening is not extreme since the amount of double-stranded RNA is small and increases during the cell cycle (Frankfurt, 1980; Shapiro, 1986). Thus, PI staining should still be useful in separating singlets versus doublets of DNA for comparison with the time-of-flight signals.

Based on the time-of-flight separation of singlets versus doublets of cells, later analyses determined that no significant differences in dye response or resting pH_i were observed between these two cell populations. Therefore, a time-of-flight selection of single cells was frequently used to improve the quality of the observed distribution of BCECF fluorescence with respect to minimizing skewness as well as keeping all data within the measuring range (while maintaining a relatively high signal). The latter point is important for analyses of cellular variability via the coefficient of variation (CV), since this parameter is extremely sensitive to any asymmetrical loss of data (e.g., off-scale data). Even using size-selected cells for data analysis, the BCECF distribution is broad (CV ~ 30–40%). This probably relates to heterogeneity in dye loading within the cells selected for study. For this reason, it would have been preferable to have used ratiometric measurements to evaluate pH_i (Montrose et al., 1987). Unfortunately, the flow cytometer was not easily configured for dual excitation, and dual emission measurements would have interfered with our (red) viability dye analysis.

We should emphasize that we have not performed a rare event analysis in our examination of cellular variability. The two techniques that have been applied to identify heterogeneity are only capable of detecting less subtle variability in the population. The first technique was to evaluate whether a significant number of cells formed a visible subpopulation on a contour plot. This technique is valuable for checking for the presence of a relatively few cells which are very different. The second technique was to evaluate the CV of the population to evaluate whether the major population was more or less variable. Thus, the CV is valuable for examining whether a relatively large number of cells are slightly different. The latter technique gave an indication of some cellular heterogeneity in the response to cellular acidification and in the rate of pH_i recovery from an acid load, but no heterogeneity could be demonstrated in the response of the cells to either PTH or to Na^+ free medium.

It was observed that the CV of BCECF fluorescence in resting cells did not decrease when cells were pH clamped (by addition of nigericin). This implies that the pH_i of the cells was as tightly distributed before, as after, imposition of the pH clamp. This is consistent with the observation of a focussing of pH_i (reduction in CV) during the pH_i recovery from an acid load, since both results imply a strict regulation of the resting intracellular pH to a value that is extremely similar between different cells (mean pH_i = 7.4–7.5). The results suggest that either the same transport mechanism is controlling pH_i in the vast majority of cells, or that different transport systems are leading to the same pH_i. Examination of the pH_i recovery in terms of Na^+ dependency and EIPA sensitivity support the former explanation, since virtually all cells behave as if Na^+/H^+ exchange (a Na^+-dependent and EIPA-sensitive process) is mediating the pH_i recovery.

When cells are acid loaded with NH_4 prepulses and allowed to recover from the acid load, a transient increase in CV is noted. Evidence from the current experiments suggest that this increase has two separable components. Since a large CV increase is seen in the virtual absence of any pH_i recovery (Na^+-free medium), the major component is likely to be due to variability in the magnitude of the cellular acid load created by an NH_4 prepulse acid load. This may relate to differences in the relative permeability of different cells to NH_3 versus NH_4 molecular forms, but could have another basis as well. The existence of a second component was initially suggested by the fact that the peak of the CV increase is observed 3–4 min after NH_4 is removed, whereas the acid load is maximal within 1 min. Since the (single cell) data analysis examines cells of similar size, this can only be explained in terms of NH_4/NH_3 permeability when some cells have drastically lower permeability to NH_3 than average. An alternate explanation is proposed, however, because in the presence of very slow transport (plus EIPA), the peak of the CV increase is observed at later times in the data collection. Thus either the NH_3 permeability is inhibited by EIPA, or the CV increase is created during the pH_i recovery. We favor the latter explanation and suggest that this minor CV increase is due to heterogeneity in the rate of pH_i recovery (Na^+/H^+ exchange), such that pH_i would tend to disperse prior to converging at the resting pH_i. Given the heterogeneity in the acid load (which produces the major CV increase), the heterogeneity in transport rate may simply relate to

different starting pH_i values. The rate of Na^+/H^+ is known to be sensitive to pH_i (Aronson, Nee & Suhm, 1982; Grinstein & Rothstein, 1986; Miller & Pollock, 1987; Moran et al., 1988).

It has been observed previously that PTH (and membrane permeant derivatives of cAMP) act to decrease the Na^+/H^+ exchange rate of both kidney cortex brush border membrane vesicles, and OK cells (Kahn et al., 1985; Pollock et al., 1986; Miller & Pollock, 1987; Moran et al., 1988). However, in both systems saturating concentrations of the hormone (or the cAMP derivative) result in an incomplete (50–60%) inhibition of the exchange rate. Since these results have been obtained from preparations that average data from an entire cell population, one possible interpretation of the data is that not all cells were responding to the hormonal agents with a decrease in Na^+/H^+ exchange. For instance, it is possible that roughly 50% of the cells were completely inhibited by PTH and that the other 50% had not been affected at all. This example would predict that, after the initial acidification, the CV should increase continually to a value at least two-fold higher than the starting CV by the end of the experiment. When this problem was addressed using the flow cytometer and OK cells, no large cellular heterogeneity (or predicted trend in CV) was observed due to the presence of a saturating concentration of PTH. This implies that the majority of the cells respond to the hormone and demonstrate an incomplete inhibition of transport.

In conclusion, OK cells (in conjunction with BCECF) are suitable for studies of intracellular pH via flow cytometry. Both singlets and doublets of cells are intact and maintain similar resting pH_i values. The population of OK cells demonstrates a recovery from an acid load which is very homogeneous with respect to its sensitivity to Na^+ removal or EIPA addition, suggesting that virtually all cells utilize Na^+/H^+ exchange for this recovery. The Na^+/H^+ exchanger is observed to focus the BCECF fluorescence (intracellular pH) of the population to a degree equivalent to pH clamping the cells with nigericin. The response of the population to PTH strongly suggests that the majority of cells respond to the hormone and that the total Na^+/H^+ exchange in individual cells is partially inhibited even in the presence of saturating PTH concentrations. This latter point would imply that the observed partial inhibition must be due to either partial inhibition of all Na^+/H^+ exchangers in a single cell or to inhibition of only some of the Na^+/H^+ exchangers in an individual cell. The results predict that a similar event may be occurring in the epithelial cells of the proximal tubule.

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