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Primary Cell Cultures From Murine Kidney and Heart Differ in Endosomal pH

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Endosomal and lysosomal pH values have been determined for many established cultured cell lines of different origins. These cell lines may be grouped into two classes based on observed differences in pH of early (recycling) endosomes. Members of the first class typically have an early endosomal pH of 6.2, whereas members of the second class typically have an early endosomal pH of 5.4. Because established cell lines may have developed artificial differences in endosomal pH due to extended culture, it remains to be determined if endosomal pH differences exist in vivo and whether they are functionally significant. To address this question, we generated adherent primary explants from mouse kidney (primarily epithelial cells) and heart (primarily fibroblasts and cardiac muscle cells). Interestingly, enhanced acidification was observed in heart cell endosomes (pH = 5.5) compared with kidney cell endosomes (pH = 6.0). These results indicate that differences in endosomal pH do not solely arise from extended cell culture and imply that such differences may be important for the proper functioning of different cell types. J. Cell. Physiol. 176:216–222, 1998. © 1998 Wiley-Liss, Inc.

Many macromolecules enter cells by the process of endocytosis. As ingested materials progress through the endocytic pathway, they are exposed to an increasingly acidic pH (Murphy et al., 1984; Mellman et al., 1986; Roederer et al., 1987). The pH of early endosomes is typically near 6, late endosomes near 5, and lysosomes even lower (for review see Mellman et al., 1986). Maintenance of an acidic intravesicular environment is essential for efficient trafficking within the cell. For example, acidification of early endosomes allows many ligands to dissociate from their receptors, whereas the highly acidic interior of the lysosome provides favorable conditions for hydrolases.

Two classes of cultured cell lines have been proposed based on their early endosomal pH (Murphy, 1988; Sipe et al., 1991). Cell lines in the first class include A549 (Cain et al., 1989), BALB/c 3T3 (Sipe and Murphy, 1987), Swiss 3T3 (Zen et al., 1992), and Chinese hamster ovary (Yamashiro and Maxfield, 1987). These cells display mild acidification of transferrin in the early (recycling) endosome (pH 6.0–6.2) followed by alkalinization during recycling. Chicken embryo fibroblasts (Killisch et al., 1992) also show this pattern. This class of cell lines is characterized by the sensitivity of their early endosomal pH to ouabain, a Na^+,K^+-ATPase inhibitor. Incubation of these cells in ouabain dramatically lowers the early endosomal pH from 6.0–6.2 to 5.3 (Cain et al., 1989; Zen et al., 1992).

The second class includes K562 (van Renswoude et al., 1982; Sipe et al., 1991), Sc9 (Sipe, 1990), and HD3 (Killisch et al., 1992) cell lines. These lines acidify transferrin in the early endosome to pH 5.4 and do not display alkalinization during recycling. Furthermore, the early endosomal pH of these lines is unaffected by ouabain, indicating that the Na^+,K^+-ATPase is not involved in early endosomal pH regulation in these cells (Sipe et al., 1991). It is interesting to note that to date, all of the members in this class are blood cell lineages: K562 and Sc9 are human and murine erythroleukemia cell lines, respectively, and HD3 is a continuous line of chicken erythroblasts. The functional significance of this correlation is unknown.

The cause of these well-documented early endosomal pH differences has yet to be identified, although theoretical modeling suggests some possible mechanisms (Rybak et al., 1997). The pH measurements discussed above, with the exception of those for chicken embryo fibroblasts, were made in immortalized cultured cell lines that have been grown for many years. Thus, differences in early endosomal pH may have arisen as an artifact of extended cell culture. For example, mutations in one of any number of genes might be expected to alter early endosomal pH. These genes include, but are not limited to, genes encoding one of the chloride channels, any of the subunits of the vacuolar H^+-

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ATPase, any of the isoforms of the Na^+,K^+-ATPase, and regulators of these genes. However, because all of the cells with a low endosomal pH are from blood cell lineages (a correlation unlikely to have arisen at random), it is at least as likely that the observations in cultured cells reflect differences existing in the intact animal which may be functionally significant.

As an initial test of the latter hypothesis, we carried out endosomal pH measurements in murine primary explants. The results indicate that at least in the two cases examined, cells with different origins display different acidification kinetics.

MATERIALS AND METHODS

Materials

FVB mice and halothane (Halocarbon Laboratories, River Edge, NJ) were generous gifts from Dr. Alan Koretsky (Carnegie Mellon University). Media 199, trypsin/ethylene diaminetetraacetic acid (EDTA) (0.25% trypsin, 1 mM EDTA), and penicillin-streptomycin solutions were obtained from Gibco BRL (Grand Island, NY). Calf serum was purchased from Intergen Company (Purchase, NY). Tissue culture dishes were obtained from Corning (Corning, NY). Fetal bovine serum was purchased from Atlanta Biologicals (Norcross, GA). Amino dextran (70,000 molecular weight) and fluorescein isothiocyanate (FITC) were purchased from Molecular Probes (Eugene, OR). All other materials were from Sigma (St. Louis, MO) unless otherwise noted.

Dextran conjugated with fluorescein isothiocyanate (FITC-dextran) was prepared as described previously (De Belder and Granath, 1973). To prepare dextran conjugated with Cy5.18.OSu (Cy5-dextran), the following procedure was used (Brockman, 1994). Amino dextran was dissolved in 0.1 M $NaHCO_3$, pH 9.5, at a final concentration of 10 mg/ml. Forty milligrams of Cy5.18-OSu (a generous gift from Dr. Alan Waggoner; see Mujumdar et al., 1993 for structure and synthesis) were dissolved in 100 μl dry dimethylformamide. The dye solution was combined with 21.67 ml of the amino dextran solution (for a molar dye-to-dextran ratio of 4:1), vortexed, and incubated 30 min at room temperature (vortexing every 10 min). The reaction was stopped by adding 3.11 mg of succinic anhydride (to block any remaining free amino groups), vortexing, and incubating for 30 min. This procedure was repeated twice for a total of three succinic anhydride incubations. To separate free dye from dextran, the mixture was passed over a G-25 Sephadex column equilibrated with PBS (140 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4). The first (dextran) peak was collected and dialyzed against distilled water overnight in Spectra/Por 12,000- to 14,000-Da cut-off dialysis tubing (Spectrum Laboratories, Inc., Laguna Hills, CA). The amount of conjugated dye was determined from absorbance at 652 nm using an extinction coefficient of 250,000 M^{-1} cm^{-1} (Mujumdar et al., 1993). The Cy5-dextran was lyophilized, weighed, and stored at −20°C. A molar dye-to-dextran ratio of 2:1 was typically achieved.

Harvesting murine cells for primary culture

Primary cultures were established essentially as described previously (Freshney, 1983). Two FVB mice (males and/or females from 2 to 5 months old) at a time were sacrificed by halothane inhalation. The mice were doused with 70% ethanol to minimize contamination of the primary cultures. Heart and kidneys were removed from both mice using scissors and forceps soaked in 70% ethanol, and as each organ was removed it was immediately placed in a 100-mm tissue culture dish containing 10 ml of sterile PBS (each organ type was placed in a separate dish). After all organs were removed, they were transferred to fresh 100-mm dishes containing 10 ml of sterile PBS. Kidneys and hearts were minced separately into 1-mm cubes using razor blades dipped in 70% ethanol. The minced tissues were transferred into sterile 15-ml conical tubes containing sterile PBS. After allowing the minced tissue pieces to settle, the PBS was aspirated, and the tissues washed once more with sterile PBS. Ten milliliters of 0.25% trypsin was added, and the tissue was incubated with rocking in a 37°C humidified 5% CO_2 incubator for 30 min. After the incubation, the minced tissue pieces were allowed to settle to the bottom of the conical tube. The supernatants, which contained dissociated cells from the tissue pieces, were collected into sterile 15-ml conical tubes by centrifugation at 800 g for 5 min. The resulting pellet containing the primary culture cells was resuspended in 5 ml of Media 199 containing 10% (vol/vol) fetal bovine serum, 1% (vol/vol) penicillin/streptomycin, and 2 mM L-glutamine and was plated into a 60-mm tissue culture dish. To the remaining pieces of tissue, 10 ml of fresh 0.25% trypsin was added, and the conical tube reincubated at 37°C for 30 min with rocking. This procedure was repeated every 30 min for 3–4 hr. Therefore, for every two mice, six to eight 60-mm dishes of primary culture cells for each organ were obtained.

The primary cultures were placed in a 37°C, 5% CO_2 humidified incubator for 24 hr to allow cells to adhere. After 24 hr the cells were washed twice with 2 ml of sterile PBS to remove non-adherent cells (i.e., blood cells) and tissue fragments. Cells were returned to the 37°C incubator in the medium described above for another 2–3 days, until they reached 60–80% confluency. After this time, cells from the same organ were trypsinized, combined, and split into the number of 60-mm tissue-culture dishes required for a single experiment (usually, 17–24). Cells required an additional 3–4 days to grow to the appropriate density for an experiment.

Culture of established cell lines

The A549 human epidermoid carcinoma cell line (Giard et al., 1973) and the human erythroleukemia cell line K562 (Lozzio and Lozzio, 1975) were grown at 37°C in a humidified 5% CO_2 incubator. A549 cells were grown in Dulbecco's modified Eagle's medium (Hyclone Laboratories, Logan, UT) supplemented with 10% (vol/vol) calf serum and 2 mM L-glutamine. K562 cells were grown in RPMI (Hyclone Laboratories) supplemented with 10% (vol/vol) heat-inactivated calf serum and 2 mM L-glutamine. Cells were plated 48 hr before the experiment so that they would be 80% confluent at the time of use.

Dextran acidification in adherent primary cultures

Acidification was measured by flow cytometry using the dual fluorochrome method (Murphy et al., 1984) with fluorescent dextran conjugates as probes of fluid-

phase endocytosis (Cain and Murphy, 1986; Roederer et al., 1987). FITC-dextran was used as the pH-dependent probe and Cy5-dextran as the pH-independent probe (Brockman, 1994). Primary cultures were grown for 3–4 days after plating as described above. Cells were 60–80% confluent on the day of the experiment. All incubations below were done directly in the culture dishes.

Preparation of experimental samples. On the day of the experiment, the medium was removed, and the cells were washed once with 37°C PBS/Ca^{2+}/Mg^{2+} (PBS containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$). Cells were incubated in 500 μl of Media 199 with or without 5 mg/ml FITC-dextran and 0.5 mg/ml Cy5-dextran in a 37°C, 5% CO_2 incubator for 3 min. The medium was removed, and the cells were washed eight times with PBS/Ca^{2+}/Mg^{2+} at 37°C to remove uninternalized dextran. These washes took 1.5–2 min to complete. Incubations were staggered so that only four to six dishes were washed at a time. (As discussed in the Results and Discussion, a control experiment using A549 cells was carried out exactly as described here except that the number of washes was varied.) After the washes, 1 ml of Media 199 (pre-equilibrated to 37°C) was added to each dish, and the cells were incubated at 37°C for 0–12 min. After the 37°C chase, cells were immediately removed from the incubator, placed on ice, and washed twice with ice-cold PBS/Ca^{2+}/Mg^{2+} to stop further endocytosis. Cells were gently scraped into 400–600 μl of PBS/Ca^{2+}/Mg^{2+} and analyzed by flow cytometry as described below.

Preparation of pH clamped samples. Cells in 60-mm dishes were washed, incubated with dextran for 3 min, and washed eight times to remove uninternalized dextran as described above. One milliliter of Media 199 was added and the cells returned to the 37°C incubator for 12 min. The medium was replaced with 400 μl of 200 mM 2-deoxyglucose, 40 mM NaN_3 in PBS/Ca^{2+}/Mg^{2+}, and the cells were incubated for 10 min at 37°C to deplete cellular ATP. After this incubation, 400 μl of pH 7.24 clamp buffer (100 mM ammonium acetate, 100 mM HEPES, 20 mM NaN_3; pH 7.24) and 80 μl of 1 M methylamine were added (directly to the 400 μl of previously added solution) and the incubation continued at 37°C for an additional 10 min to completely equilibrate pH. After this incubation, cells were removed from the incubator and placed on ice. Cells were directly scraped into the clamp buffer mixture and analyzed by flow cytometry as described below.

Calculation of average intravesicular pH. Samples were prepared in duplicate (experimental samples) or triplicate (pH 7.24 clamped samples). A mean fluorescence value was calculated for each fluorochrome for each sample and autofluorescence values calculated from unlabeled samples were subtracted (autofluorescence composed 7–10% of the total signal). The average of the mean fluorescence values for FITC-dextran and Cy5-dextran were calculated for the replicates of each time point and the pH 7.24 clamped samples. For each time point, the ratio of these FITC and Cy5 averages was calculated, normalized to the ratio for the pH 7.24 clamped condition, and extrapolated to the standard curve (see below) to determine an intravesicular pH.

Generation of standard curve

Before performing acidification measurements, a standard curve was generated for cells labeled with fluorescent dextrans. To conserve primary cells, established cell lines were used for generating the standard curve. A549 and K562 cells were labeled, washed, chased, and clamped as described above for primary cells except that clamp buffers (see above) adjusted to various pH values were used. Cells were then placed on ice, scraped, and analyzed by flow cytometry as described below. Triplicate samples were used for each pH.

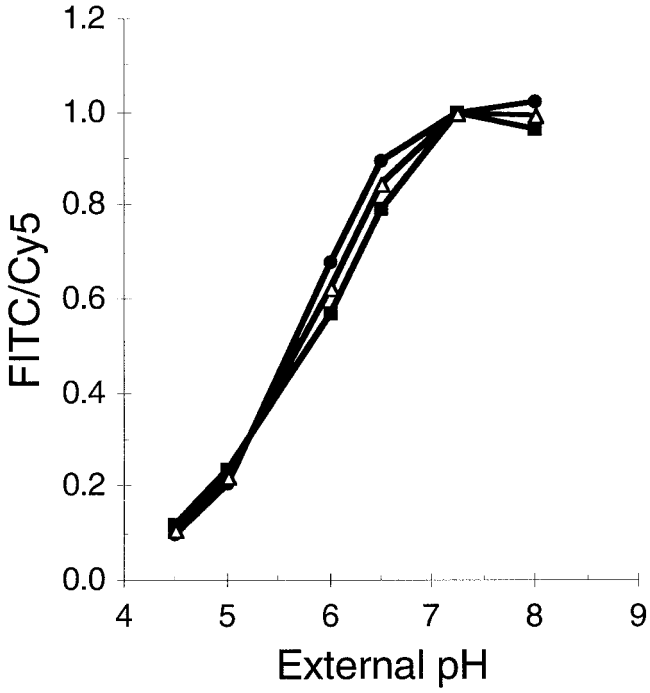

Fig. 1. Standard curves for dextran acidification experiments. K562 (filled squares) or A549 (filled circles) cells were labeled with FITC- and Cy5-dextran as described in Materials and Methods. Samples were clamped at specific pH values and analyzed by flow cytometry. Average FITC and Cy5 fluorescence values were calculated for triplicate samples after correction for autofluorescence. The ratio of these averages was calculated for each pH and normalized to pH 7.24. The average of the values for both cell types was also calculated (open triangles).

For the generation of the standard curve, it was important to ensure that all endocytic compartments are clamped to the desired pH and remain at that pH during analysis. To prevent the pH of the clamped sample from being altered due to mixing with the sheath fluid of the flow cytometer, sheath buffers (PBS, 0.05% NaN_3) matched in pH to the pH of the clamped samples were used. This matching required that the sheath fluid be changed for each set of clamped pH samples. After each change, the pH of the sheath fluid exiting the nozzle was monitored until it was determined that it equaled the pH of the samples to be analyzed.

Mean fluorescence values were calculated as above and corrected for autofluorescence (which composed 1–3% of the total signal). The ratio of the average FITC and Cy5 fluorescence values was calculated for the replicate samples at each pH and normalized to 1 at pH 7.24 (Fig. 1). A standard curve was generated for both A549 (adherent) and K562 cells (nonadherent). The average of these two curves was used for conversion of

measured fluorescence ratios to pH. The similarity of the curves obtained for different cell types supports the use of the average curve for calculating pH values from primary cultures.

Flow cytometry

An Epics Elite (Coulter Corporation, Miami, FL) was used for all experiments. Samples were kept on ice during analysis. A forward scatter threshold was used to select events to be recorded in list mode (typically 10,000 events per sample). Green (FITC) fluorescence was measured using 488 nm excitation (15 mW) and a 525-nm band-pass filter (25-nm bandwidth). Far Red (Cy5) fluorescence was measured using 633-nm excitation (10 mW) and a 675-nm band-pass filter (25-nm bandwidth). Mean values for the parameters for single viable cells were calculated using appropriate forward and side scatter windows.

RESULTS AND DISCUSSION

Using flow cytometry to measure endosomal pH

Several methods have been used to measure the pH of endocytic vesicles. These methods include fluorescence microscopy (Yamashiro et al., 1983, 1984; Yamashiro and Maxfield, 1987; Zen et al., 1992), fluorescence spectroscopy (Ohkuma and Poole, 1978; Geisow et al., 1981; van Renswoude et al., 1982), and flow cytometry (Sipe and Murphy, 1987; Cain et al., 1989; Sipe et al., 1991; Killisch et al., 1992). Flow cytometry has several advantages over the other methods, which include (1) the ability to eliminate dead cells from the final analysis, (2) the ability to analyze several thousand individual cells in a short time, (3) high signal:noise ratios due to integration of all cellular fluorescence and the use of laser light sources, and (4) absence of problems with photobleaching because each cell is only analyzed once (for review see Murphy and Roederer, 1986; Murphy, 1989).

Measurement of intravesicular pH using the pH-sensitive dye fluorescein was pioneered by Ohkuma and Poole (1978). This technique takes advantage of the fact that at 495-nm excitation FITC is pH sensitive, whereas at 450 nm excitation, it is not. Therefore, the 495/450 ratio can be converted to a pH value independent of the amount of FITC present. Subsequently, the dual fluorochrome technique for measuring intravesicular pH was developed (Murphy et al., 1984). In this method, a mixture of pH-sensitive (most commonly FITC) and pH-insensitive (such as Cy5 or a rhodamine derivative) conjugates are used for labeling intravesicular compartments. This method provides a better dynamic range than the single fluorochrome method, and has been extensively used with various endocytic markers (e.g., dextran, transferrin) in conjunction with flow cytometry (Roederer et al., 1987; Sipe and Murphy, 1987; Cain et al., 1991) and confocal microscopy (Johnson et al., 1993; Presley et al., 1993; van Weert et al., 1995).

Previous pH measurements from our laboratory used jet-in-air interrogation, in which excellent laminar flow was observed and excellent standard curves could be obtained simply by adjusting the pH of the sample buffer. Using the Epics Elite cytometer with a quartz cuvette with a 100-μm nozzle, mixing between the sheath buffer and pH clamped samples resulted in improper standard curves (data not shown). The problem was easily corrected by either preparing the sheath fluid without buffers (data not shown) or by matching the pH of the sheath fluid to the pH of the clamped samples (Fig. 1).

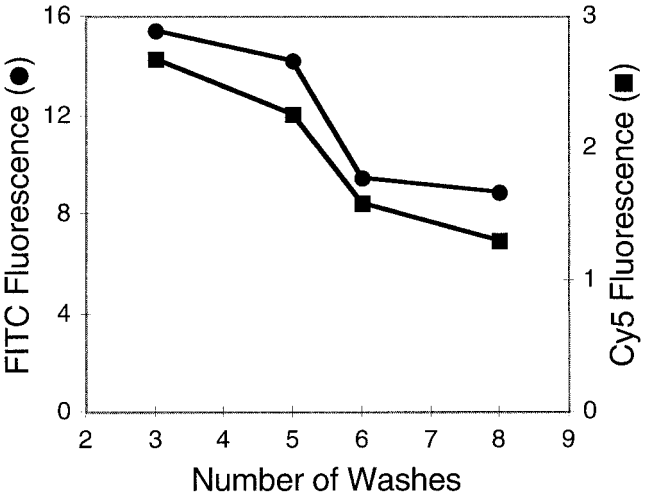

Fig. 2. Determination of optimal number of washes after dextran labeling. A549 cells were labeled with FITC- and Cy5-dextran for 10 min at 37°C as described in Materials and Methods. Cells were subsequently washed the indicated number of times with 37°C PBS/Ca^{2+}/Mg^{2+}. The average FITC and Cy5 fluorescence values were calculated for each condition, corrected for autofluorescence for that fluorochrome from unlabeled cells, and expressed relative to autofluorescence. Data are from one experiment with duplicate samples.

Optimization of dextran acidification procedures

Our goal was to monitor the kinetics of acidification of a fluid-phase probe in early endocytic compartments. By using FITC- and Cy5-dextran at the concentrations indicated in the Materials and Methods, it was determined that the minimal incubation time at 37°C required to generate an acceptable signal-to-noise (fluorescence-to-autofluorescence) ratio was 3 min (data not shown). For all subsequent experiments, cells were labeled for 3 min and then washed to remove unincorporated dextran. We have observed in previous work that washing is ineffective if plates are placed on ice (perhaps due to the viscosity of concentrated dextran solutions at low temperatures). Therefore, when removing nonendocytosed dextran, we washed with PBS/Ca^{2+}/Mg^{2+} at 37°C. To determine the optimal number of washes, we performed a test using A549 cells. Increasing the number of washes decreased the cell-associated fluorescence, with a minimum being reached after six to eight washes (Fig. 2). The decrease can be attributed to the removal of surface-adhered dextran as well as exocytosis (retroendocytosis) of some dextran. We chose to use eight washes, which required 1.5–2 min to perform for four to six plates of cells. Therefore, the earliest time point for which we determined an intravesicular pH was 5 min, the time typically required on average for material to reach the early (recycling) endosome. Measurements were also made for cells further incubated at 37°C to chase the dextran into later endocytic compartments.

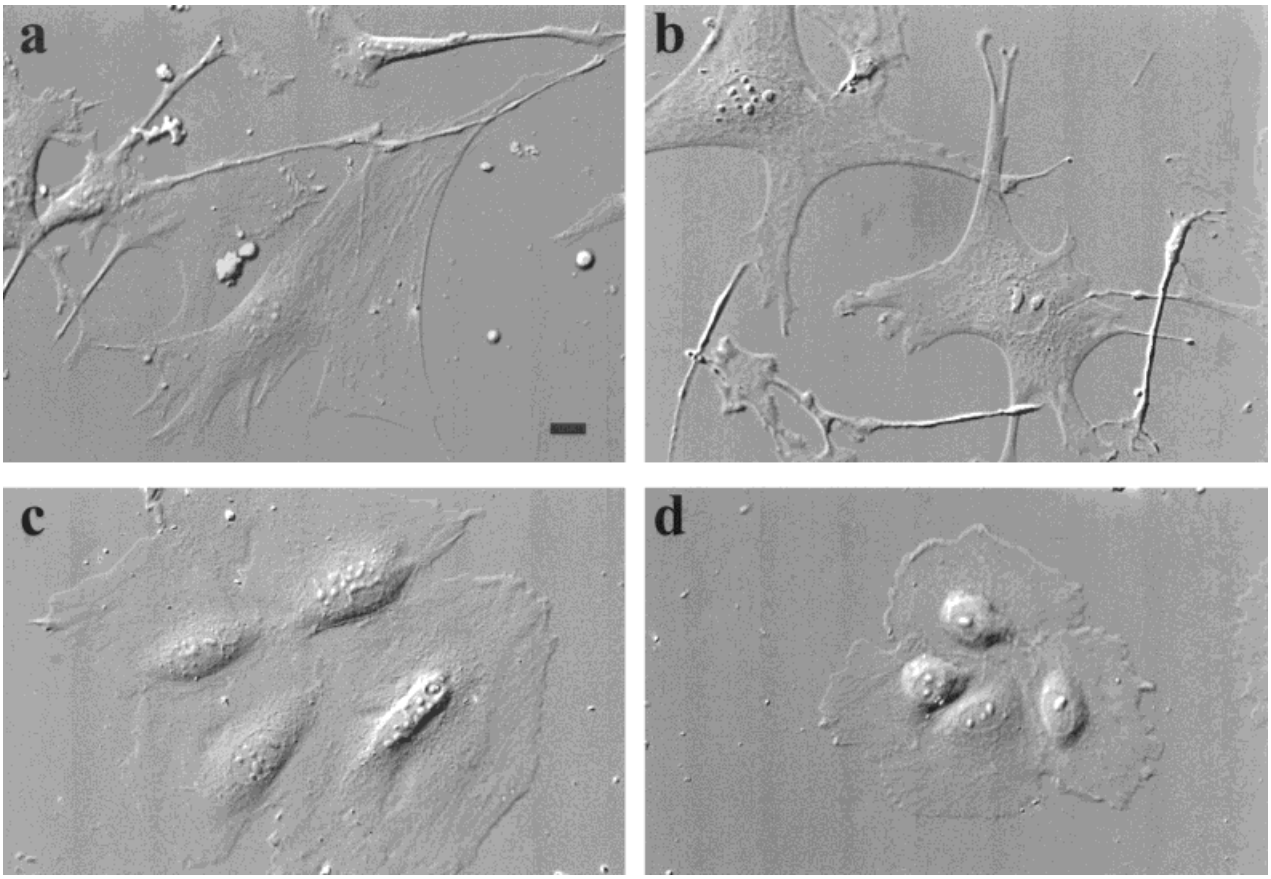

Fig. 3. Cell morphologies of primary cultures. Cells from primary cultures were plated on collagen-coated coverslips at the same time that cells were plated for measurement of acidification. Two days later they were examined by differential interference contrast (DIC) microscopy. Images were collected digitally and corrected for non-uniform illumination by flat-field division. **a,b:** Cells cultured from heart. Note the many actin filaments and protrusions in the larger cells, and the occasional spindle-shaped cells. **c,d:** Cells cultured from kidney. Note the rounded morphology. Bar: 10 μm.

Endosomal pH differences between heart and kidney primary cultures

For each experiment, primary cells were isolated from two mice from 2 to 5 months of age. We chose tissues that are fairly homogenous in cell type and which grew well in culture. Figure 3 shows micrographs of cells representative of each culture. Some heterogeneity is expected in such primary cultures, but the kidney cultures consisted largely of cells with morphology consistent with subconfluent epithelial cells. The cultures from heart appeared to consist primarily of myocytes and fibroblasts.

Figure 4 shows calculated intravesicular pH values for both heart and kidney primary cells. There does not seem to be a significant difference in endosomal pH at early time points, with both cell types acidifying the endocytosed dextran to approximately pH 6. However, at later time points heart cells consistently had a lower intravesicular pH than did kidney cells. As described in the Introduction, the division of cell lines into two classes based on endosomal pH measurements has been proposed (Murphy, 1988; Sipe et al., 1991). The results in Figure 4 are reminiscent of the comparison of transferrin acidification between A549 and K562 cells, in which acidification over the first 5 min is similar for the two cell types, but K562 cells acidify further over the subsequent 2–5 min (Sipe et al., 1991). However, in the case of transferrin (which remains membrane bound during its internalization and recycling), significant alkalinization is observed in A549 cells after the minimal pH of 6.2 is attained. This finding is not observed for fluorescent dextran conjugates, which remain in the lumen of endocytic compartments. The measurements reported here represent averages over all compartments containing endocytosed fluorescent probes and a pulse-chase protocol is used to limit the number of those compartments. The most straightforward interpretation of the results is that the pH in endosomes from cells isolated from heart is regulated differently than it is in cells isolated from kidney. However, the differences in the average pH observed between the primary cultures might also arise from differences in the kinetics of endocytic traffic through compartments of different pH.

Endosomal pH has not been monitored in intact cells for anything but cultured cell lines, with one exception. An early endosomal pH of 5.4 was calculated for both rabbit reticulocytes and nucleated erythroid cells from

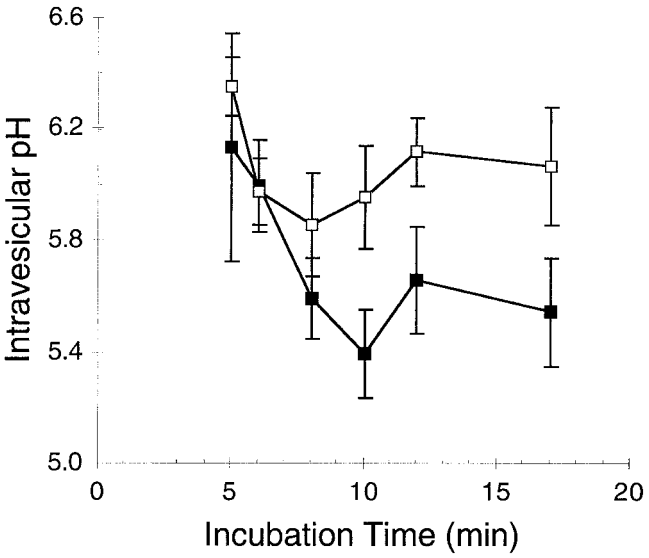

Fig. 4. Comparison of dextran acidification in primary cultures from heart and kidney. Primary cultures were obtained from mouse heart (filled squares) or kidney (open squares) and dextran acidification was measured as described in Materials and Methods. The ordinate shows total time of incubation at 37°C from dextran addition: pulse of 3 min, wash of 2 min, and chase of 0–12 min. Values shown are the average and standard error of the mean for five to seven experiments.

fetal rat liver (Paterson et al., 1984), consistent with previous reports that cultured cell lines of erythroid origin have a low early endosomal pH (van Renswoude et al., 1982; Sipe, 1990; Sipe et al., 1991; Killisch et al., 1992). Clearly, more information is needed on the pH of endocytic compartments in cells from intact tissues or animals. Measurements of acidification in isolated organelles or cell homogenates are of limited use here because values are usually not converted to pH in such experiments, and the pH values obtained when calibration is performed often do not correspond with those measured in living cells (Gaete et al., 1991; Van Dyke, 1993).

The results of Figure 4 demonstrate that the division of cells into classes based on endosomal pH can be extended to include primary cultures of different origins, although how many different endosomal acidification classes exist and whether they are the same for transferrin and dextran are currently unanswered questions. It will be of interest to determine whether endosomal pH differences exist between cells from other tissues. The use of antibodies to cell surface markers would also allow determination of endosomal pH for specific cell types within explants. The functional significance of endosomal pH variation between cells of different origins remains unknown. Ultimately, it will be important to determine whether the maintenance of a specific endosomal pH is required for differentiated cells to carry out their specialized functions.

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