Regulation of intracellular pH in subpopulations of cells derived from spheroids and solid tumours

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Summary Solid tumours are known to develop regions of extracellular acidity and survival of tumour cells in such regions depends on membrane-based mechanisms which regulate intracellular pH (pHᵢ). We have therefore developed a method, based on dual staining of cells and flow cytometry, to study the regulation of pHᵢ in subpopulations of tumours and spheroids. The activity of membrane-based pHᵢ regulating transporters was studied in EMT-6 and MGH U1 cells grown in monolayer culture, spheroids, and tumours. pHᵢ was measured with the fluorescent pH probe 2′,7′-bis-(2-carboxyethyl)-5-(and-6)carboxyfluorescein, and Hoechst 33342 was used to identify cells from different regions of tumours and spheroids. In monolayer culture, incubation of cells for 18 h at pH 6.6 led to a 1.3–1.5-fold enhancement in the activity of both the Na⁺/H⁺ exchanger and the Na⁺+-dependent Cl⁻/HCO₃⁻ exchanger. This effect was prevented by the protein synthesis inhibitor cycloheximide. Cells from the centre of EMT-6 spheroids had increased activity of the Na⁺/H⁺ exchanger compared to cells from the periphery, when spheroids were grown in medium at pH 6.6, but not at pH 7.4. By contrast, in MGH U1 spheroids, cells from the centre had increased activity of the Na⁺/H⁺ antiport under both sets of conditions. There was no significant difference in the activity of the Na⁺/H⁺ exchanger in cells derived from different subpopulations of EMT-6 tumours or MGH U1 xenografts in nude mice. Although upregulation of Na⁺/H⁺ exchange occurs after exposure to acidic conditions in vitro, the microenvironmental conditions found within solid tumours do not appear to cause this effect. Our results suggest the feasibility of pharmacological inhibition of Na⁺/H⁺ exchange activity as an approach to therapy directed against nutrient-deprived tumour cells.

Spontaneous cell death has been observed to occur commonly within regions of solid tumours. Although the causes of spontaneous cell death within tumours are not known, the poorly developed vasculature may contribute to this process, by failing to provide adequate nutrients or to remove catabolites (Vaupel et al., 1989). As a result, tumours may contain regions of hypoxia and reduced extracellular pH (pHₑ), and this combination may be responsible, in part, for the cell death which occurs (Rotin et al., 1986). Although microelectrode measurements have revealed consistently that the average pHₑ within solid tumours is approximately 0.5 pH units lower than that in normal tissues, techniques that measure predominantly intracellular pH (pHᵢ), such as ³¹P nuclear magnetic resonance spectroscopy, suggest similar values of pHᵢ in tumours and normal tissues (Vaupel et al., 1989). These observations imply that tumours cells are able to regulate pHᵢ under the acidic conditions encountered within solid tumours.

Three major mechanisms allow cells to regulate their pHᵢ under acidic conditions. These are the buffering capacity of the cytosolic and organellar contents, and two membrane-based transport systems, the Na⁺/H⁺ exchanger and the Na⁺+/H⁺ exchanger. The buffering capacity of a cell is its ability to buffer a change in pHᵢ, following the addition (or removal) of H⁺, and is comprised of both bicarbonate-dependent and non-bicarbonate (mainly protein) components (Roos & Boron, 1981; Boron, 1989). Intracellular buffering provides substantial protection for the cell against effects of an acid load, with most cells capable of buffering millimolar concentrations of H⁺ (compared to the micromolar concentrations that are normally present) (Roos & Boron, 1981).

The Na⁺/H⁺ exchanger is a membrane based transport mechanism that is ubiquitous in mammalian cells. The exchanger is a 110 kD protein whose gene has been cloned from several tissues in different species (Sardet et al., 1989; Fliegel et al., 1991; Hildebrandt et al., 1991; Reilly et al., 1991; Tse et al., 1991). It uses the inwardly directed Na⁺ gradient to pump H⁺ out of cells, and its operation is inhibited by amiloride and its analogs (L’Allemain et al., 1984; Grinstein & Rothstein, 1986). Chronic exposure of cultured renal cells to acidic extracellular conditions has been shown to increase the activity of the Na⁺/H⁺ exchanger by causing the synthesis of new exchange proteins in a process that is dependent on protein kinase C and inhibited by cycloheximide (Horie et al., 1990; Horie et al., 1992).

The other major transporter that regulates pHᵢ is the Na⁺+-dependent HCO₃⁻/Cl⁻ exchanger. This exchange has been detected in most but not all cell lines tested (Reinertsen et al., 1988; Tonnesen et al., 1990). It employs the inwardly directed Na⁺ gradient to exchange intracellular Cl⁻ for extracellular HCO₃⁻, and is inhibited by the stilbene derivative 4,4′-didiethoxyanilinostilbene-2,2′-disulfonic acid (DIDS) (Cassel et al., 1988; Reinertsen et al., 1988). Little is known of its structure and molecular biology. Although chronic acidosis in vivo has been shown to increase the activity of renal HCO₃⁻ exchangers, the mechanisms have not been characterised (Grassl, 1991).

Previous studies in tissue culture have suggested that under the microenvironmental conditions that exist within the acidic regions of solid tumours, the Na⁺/H⁺ exchanger is likely to be responsible for the majority of regulation of pHᵢ (Boyer & Tannock, 1992). Agents that inhibit the operation of this exchanger show considerable potential for causing pHᵢ-dependent cytotoxicity and thus selective killing cells in the acidic regions of solid tumours (Tannock & Rotin, 1989; Newell et al., 1992; Maidorn et al., 1993). Rational development of these agents requires an understanding of how regulation of pHᵢ by tumour cells may be modified by the acidic milieu of tumours. We have examined therefore the operation of the Na⁺/H⁺ exchanger in tumour cells under chronic acidic conditions in culture, and have developed a flow cytometric method to extend these observations to the study of pHᵢ regulation in different subpopulations of spheroids and experimental tumours.

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Materials and methods

Cells
Experiments were performed with murine EMT-6 cells (obtained from Dr R. Sutherland, University of Rochester, NY, USA), and the human bladder carcinoma cell line MGH U1 (obtained from Dr G. Prout, Massachusetts General Hospital, Boston, MA, USA). Cells were maintained routinely in a medium with 5% foetal calf serum (FCS), and new cultures, free of Mycoplasma, were re-established from frozen stock every 3 months.

Reagents
Ethylisopropyl-amiloride (EIPA) was synthesised by Aldrich (Milwaukee, WI, USA), as described previously (Cragoe et al., 1967). DIDS was purchased from ICN Biochemicals (St Laurent, PQ, Canada). 2'7''-bis-(2-carboxyethyl)-5-(and-6)carboxyfluorescein (BCECF) acetoxyethyl ester was purchased from Molecular Probes (Eugene, OR, USA). All other reagents were obtained from Sigma (St Louis, MO, USA).

Solutions
Unless otherwise indicated, all solutions were nominally HCO$_3^-$ free. NaCl solution contained 140 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM CaCl$_2$, 1 mM MgCl$_2$, buffered to the indicated pH with 20 mM MES/Tris. NaHCO$_3$ solution contained 25 mM NaHCO$_3$, 115 mM NaCl, and other components identical to those in NaCl solution. All solutions containing NaHCO$_3$ were prepared in advance, but without the NaHCO$_3$, this was added immediately before use. N-Methyl-D-glucamine (NMG) and KCl solutions were prepared by iso-osmotic replacement of NaCl with NMG and KCl respectively; the other components were identical to those described above for NaCl solution.

pH medium was prepared by adding 20 mM MES to regular medium and adjusting to the desired pH with HCl or NaOH. Medium containing HCO$_3^-$ was adjusted to the desired pH by using HCl or NaOH. Prior to use, it was bubbled with 5% CO$_2$ for 2 h and the pH was then re-adjusted. By following this procedure, the pH of medium remained within 0.1 pH units of the desired value for up to 24 h.

Evaluation of pH, and its regulation for cells in monolayer
Cells grown as a monolayer on glass coverslips were exposed to 2 µg ml$^{-1}$ of the acetoxyethyl ester of BCECF in serum free α-medium at 37°C for 20 min. The coverslip was then placed into a cuvette using a specially designed holder aligned at an angle of 30° to the excitation beam of a Perkin Elmer LS3 fluorometer (Perkin Elmer, Mississauga, Ontario). The holder also served as a cap for the cuvette, minimising the loss of CO$_2$. The cuvette was equipped with a perfusion system to allow exchange of the buffer surrounding the cells. Exchanges were made with a volume of buffer at least ten times greater than the volume contained within the cuvette. The temperature of the solution in the cuvette was controlled precisely and all experiments were carried out at 37°C.

Within the range of pH 6.0–7.5, fluorescence intensity of BCECF at 525 nm (following excitation at 495 nm) is linearly related to pH (Rink et al., 1982). At three time points during each experiment (prior to intracellular acidification, following intracellular acidification, and at the end of the experiment), fluorescence intensity was measured at the same emission wavelength but with an excitation wavelength of 440 nm (Schwartz et al., 1990). Following excitation at this wavelength, fluorescence intensity is independent of pH, and depends only on the amount of BCECF present. The ratio of fluorescence intensity at pH-dependent and independent wavelengths provides therefore an estimate of pH, that is independent of the amount of BCECF present in cells or of loss of cells from coverslips. Calibration of fluorescence measurements was performed using the ionophore nigericin, in a solution containing 140 mM K$^+$, as described previously (Thomas et al., 1979).

Intracellular acidification was achieved by placing cells in KCl solution containing NH$_4$Cl for 30 min. Acidification to a level determined by the concentration of NH$_4^+$ used, was then produced by exchanging the NH$_4$Cl containing solution with a NH$_4$Cl-free solution (Boron, 1989).

Cytoplasmic acidification was carried out in Na$^+$ and HCO$_3^-$free buffer. In experiments designed to measure the activity of the Na$^+$/H$^+$ exchanger, this buffer was replaced after intracellular acidification by Na$^+$-containing, HCO$_3^-$free buffer, pH 7.4. By contrast, in those experiments where the action of the Na$^+$-dependent Cl$^-$/HCO$_3^-$ exchanger was investigated, the buffer was replaced by Na$^+$ and HCO$_3^-$-containing buffer, pH 7.4, with 10 µM EIPA (which provides inhibition of Na$^+$/H$^+$ exchange activity). The combined activity of both exchangers was evaluated by exchanging with Na$^+$ and HCO$_3^-$-containing buffer, pH 7.4, in the absence of EIPA. Following the change in extracellular buffer, the maximal rate of the resulting intracellular alkalisation was measured by the fluorometer. The results of these experiments were converted into H$^+$ efflux by multiplying the observed rates of change of pH by the total buffering capacity of the cells (see below).

In some experiments, we assessed the influence of chronic exposure to low levels of pH, on the activity of the pH-regulating exchangers. For these experiments, cells growing on glass coverslips were placed into pH adjusted medium at varying times prior to the evaluation of the membrane-bound exchangers which regulate pH. Measurement of the activity of the exchangers was carried out in buffers with pH 7.4.

Calculation of intracellular buffering capacity
Buffering capacity is the capacity of a cell to buffer changes in pH, following addition or removal of H$^+$; it is defined as the ratio of moles of H$^+$ (or OH$^-$) added to the resulting change of pH, i.e. Δ[H$^+$]/ΔpH (Roos & Boron, 1981). In order to measure intrinsic (non-bicarbonate) intracellular buffering capacity, cells were exposed to HCO$_3^-$free NMG solution containing 3 mM ammonium chloride for 5 min, followed by replacement of the extracellular fluid with NH$_4$-free NMG solution. The resulting fall in pH was measured and used to calculate intrinsic buffering capacity using the formula described previously (Boyer & Tannock, 1992). Measurements of intrinsic buffering capacity were made at the resting level of pH, only, since values have been shown to be constant over the range of pH 6.4–7.2 (Grinstein et al., 1984). Bicarbonate buffering capacity was calculated as 2.3 [HCO$_3^-$] (Boron, 1989). The value of [HCO$_3^-$] was calculated from knowledge of pH, p$_{CO_2}$ and [HCO$_3^-$]. The total buffering capacity is the sum of intrinsic buffering capacity and bicarbonate buffering capacity.

Spheroids
Some experiments were performed with multicellular tumour spheroids. Spheroids provide a tissue culture model for tumours where cells exist within a variable microenvironment (Sutherland, 1988). MGH U1 spheroids were grown from a subline of MGH U1 cells, as described previously (Erlchman & Tannock, 1986). They were maintained routinely in spinner flasks containing HEPES-buffered, HCO$_3^-$-free medium supplemented with 10% FCS. EMT-6 spheroids were grown by seeding EMT-6 cells into uncoated Petri dishes, and allowing them to grow overnight as described previously (Newell et al., 1992). The following day, the small spheroids that had formed were placed into spinner flasks with HEPES-buffered, HCO$_3^-$-free medium and 15% FCS. Medium was exchanged daily thereafter.

Spheroid populations were obtained by suspension of spheroids to the fluorescent dye Hoechst 33342 followed by dissociation and flow
cytometry (Durand, 1982). Spheroids (diameter 500–600 μm) growing in a spinner flask were exposed for 20 min to Hoechst 33342 (0.5 or 1 μM for EMT-6 and MGH U1 spheroids respectively). They were then rinsed three times in ice-cold PBS and dissociated using a combination of trypsin and gentle mechanical dissociation.

**Growth of tumours**

MGH U1 bladder carcinoma and EMT-6 mammary sarcoma were grown in inbred female Swiss Nude (Taconic; Germantown, NY, USA) and Balb/c BYJ (Jackson Laboratories; Bar Harbor, Maine, USA) mice respectively. Tumours were initiated by injecting 2.5–5 × 10⁶ cells into the left hind leg. Growth of the tumours was monitored by passing the leg through a strip of lucite with graded circular holes. The diameter of the tumour-bearing leg was converted to an estimate of tumour weight using a previously defined calibration curve. Tumours were used for experiments when they had attained a weight of 0.3–0.5 g (approximately 9 days of growth).

Subpopulations of tumour cells at different distances from functional blood vessels were obtained by administration of Hoechst 33342, followed by tumour excision, dissociation and flow cytometry (Chaplin et al., 1985). In Balb/c mice a 30 min infusion of Hoechst 33342 (1 ml of a 1 mg ml⁻¹ solution in water) was administered via the lateral tail vein. We were unable to perform tail vein infusions consistently in nude mice and in these animals an intraperitoneal injection of 1.5 mg of Hoechst 33342 in 0.75 ml of water was used. Thirty minutes after injection (or at the conclusion of the infusion) mice were killed by cervical dislocation and a tumour excised. The tumour was placed into ice-cold PBS and minced into fine pieces using crossed scalpel blades. A single cell suspension was produced by treatment with trypsin and DNAase 1 for 10–15 min (at 37°C) followed by passage through a fine screen.

**Potential for evaluation of pHi from dissociated spheroids and tumours**

We wished to adapt a flow cytometric method to measure pHi in cells from subpopulations of spheroids and tumours (Hedley & Jorgensen, 1989). Initially cultured EMT-6 and MGH U1 cells were used to model the changes in pHi that might occur during sample preparation. Following trypsinisation, cells were suspended in serum-free α-medium and exposed to BCECF-AM, 2 μg ml⁻¹ for 20 min, at 37°C. In order to set the level of pHi, to either 6.6 or 7.2, the cells were then centrifuged and resuspended in KCl solution at pH 6.6 or 7.2, which contained nigericin, 2 mg ml⁻¹. After 5 min, the cells were centrifuged once more, and resuspended in room-temperature NMG buffer containing 10 μM EIPA at various levels of pHi; this solution provides complete inhibition of Na⁺/H⁺ and Na⁺/H⁺-dependent HCO₃⁻/Cl⁻ exchangers that regulate pHi under acidic conditions. The cell suspensions were then placed into two cuvettes in an Amino-Bowman Series 2 Fluorometer (SLM, Urbana, IL, USA), this machine allows rapid alternation of fluorescence measurements from two cuvettes and pHi was monitored, using the ratio method described earlier, by alternating between each of the cuvettes for up to 60 min.

**Evaluation of pHi, and its regulation in dissociated spheroids and tumours**

Regulation of pHi in cells derived from spheroids or tumours was assessed by flow cytometry. The instrument used was a Coulter Epics Elite flow cytometer (Coulter Electronics, Hialeah, FL, USA), equipped with air-cooled HeCd (325 nm) and argon (488 nm) lasers, and modified to allow constant control of sample temperature via a circulating water bath. The level of pHi was measured by the ratio of the 525 nm (pH dependent) and 640 nm (pH independent) emissions of BCECF following excitation at 488 nm. Calibration of fluorescence ratio measurements was performed using the ionophore nigericin, in a solution containing 140 mM K⁺, as described previously (Thomas et al., 1979). Hoechst fluorescence was measured at 450 nm following excitation at 325 nm.

A single cell suspension was prepared from tumours or spheroids as described above. After exposure to the 2 μM ml⁻¹ of the acetoxymethyl ester of BCECF in serum-free α-medium for 20 min at 37°C, cells were resuspended in NMG buffer. Intracellular acidification was produced by exposure to nigericin for 3–4 min (spheroids) or by exposure to and subsequent removal of 10 mM NH₄Cl (tumours); cells were then centrifuged. There was no difference in the acidification produced, or subsequent activity of the Na⁺/H⁺ exchanger following use of the two different methods of acidification (data not shown). Recovery from acidification was measured following resuspension of the pellet in sodium buffer.

Flow cytometric measurements of intrinsic buffering capacity were made in a manner analogous to that described above for cells growing in monolayer. However, rather than measuring the fall in pHi following removal of NH₄Cl, the immediate increase in pHi resulting from exposure of cells to 5 mM NH₄Cl was determined. The measurements and calculations were otherwise identical.

Regulation of pHi, in different spheroid and tumour subpopulations was assessed by cell separation based on the intensity of Hoechst staining of the cells (Durand, 1982; Chaplin et al., 1985). In our experiments, cells were divided into three populations, representing the brightest 25% of cells, the dimmest 25% of cells, and the intermediate 50%. The rate of change of pHi, after addition of sodium was then determined for each of these subpopulations, by calculating the average pHi over 1 min intervals, for 8–10 min. The mean values of different subpopulations were compared using Student’s t-test.

**Identification and gating of tumour cells**

Because tumours contain a variety of host and stromal cells it was necessary to identify the tumour cells prior to measurement of pHi. This was achieved by gating the sample on the basis of forward and 90° light scatter which provides an indirect measure of cell size. Since the tumour cells were larger than the normal host cells these measurements could be used to identify tumour cells. To assess the effectiveness of this gating procedure we measured also the ploidy of the cells; both the EMT-6 and MGH U1 tumours are aneuploid while the host cells are diploid. A parallel sample was stained with 10 μM Hoechst 33342 for 30 min, to obtain a DNA histogram. In the ungated population of cells, typically 40–60% of the cells were aneuploid. Following gating as described above, 85–90% of the cells were aneuploid, and only these cells were included in the measurement of pHi. The excluded population contained 5–10% aneuploid cells.

**Irradiation and sorting of tumours**

In order to ensure that our separation procedure resulted in selection of cells from hypoxic (and presumably acidic) regions of tumours, we assessed the survival of different tumour subpopulations following irradiation in vivo. Immediately after intraperitoneal injection of Hoechst 33342, unanaesthetised and unrestrained tumour-bearng mice were irradiated with a dose of 15 Gy using a ¹³⁷Cs source at a dose rate of 0.58 Gy min⁻¹. Following this, the mice were killed, and the tumours excised and prepared for flow cytometry as described above. Cells were sorted on the basis of fluorescence intensity of Hoechst 33342 into three populations identical to those in the pHi regulation experiments described above. The sorted cells were incubated in culture dishes containing α-medium, in a humidified atmosphere containing 5% CO₂ at 37°C for 12 days. The number of colonies containing >50 cells was then counted. The surviving fraction was expressed relative to that of unirradiated controls.
Measurement of pH, in vivo

Mice were anesthetised with sodium pentobarbital (M.T.C. Pharmaceuticals; Cambridge, Ontario), 65 mg kg⁻¹ body weight. Measurements of pH₆ were made using a miniature glass electrode which has a sensing area of diameter 500 μm (model MI-408b, Microelectrodes Inc; New Hampshire) against a silver–silver chloride reference electrode (model MI-402, Microelectrodes Inc; New Hampshire) using a high impedance pH meter (PHM 82, Radiometer, Copenhagen). The reference electrode was inserted subcutaneously on the back, and bathed in phosphate buffered saline. The pH microelectrode was inserted directly into the tumour or muscle tissue after the overlying skin had been removed. Measurements of tumour pH₆ were made at increments of 50–75 μm along a single track at a depth of 200–500 μm into the tumour using a specially designed micromanipulator. At least four measurements were made per tumour.

Results

Effects of low pH, exposure on regulation of pH₆

Initial experiments were undertaken to determine whether the activity of the Na⁺/H⁺ exchanger and the Na⁺-dependent Cl⁻/HCO₃⁻ exchanger were altered by growth of cells under acidic conditions. Cells were grown for up to 18 h at levels of pH₆ in the range 6.0 to 7.2. Under these conditions, clonogenic survival of the cells was not altered, and cells grew normally (data not shown). There was a time and pH₆ dependent increase in the combined activity of the Na⁺/H⁺ exchanger and the Na⁺-dependent Cl⁻/HCO₃⁻ exchanger in MGH U1 cells (Figure 1). The maximum increase, a 1.6-fold enhancement of activity, occurred after 18 h incubation at pH₆ 6.6; evaluation after longer times was not undertaken because of loss of cellular viability. Similar results were obtained with EMT-6 cells (Table 1).

The apparent increase in the combined activity of the Na⁺/H⁺ antiport and the Na⁺-dependent Cl⁻/HCO₃⁻ exchanger could have been due to changes in the intrinsic buffering capacity of the cell after low pH₆ exposure. We therefore measured intrinsic buffering capacity after MGH U1 cells had been growing at pH₆ 6.6 for 18 h. There was no difference between these values (38.7 ± 4.0 mm H⁺/pH unit (mean ± s.e.m. of four experiments)) and control values (39.3 ± 3.8 mm H⁺/pH unit). This result implies that the observed changes were due to alterations in either the number or activity of the exchangers.

The observed increase in the combined activity of the Na⁺/H⁺ exchanger and the Na⁺-dependent Cl⁻/HCO₃⁻ exchanger may have been due to an increase in the activity of both of the exchangers or only one of them. We therefore repeated the experiments in the absence of HCO₃⁻ (to measure activity of the Na⁺/H⁺ exchanger) or in the presence of EIPA (to measure activity of the Na⁺-dependent Cl⁻/HCO₃⁻ exchanger). Table 1 shows that the rate of pH₆ recovery due to each of the two exchangers was increased by acid incubation.

In order to determine whether the increase in the activity of the exchangers was dependent on protein synthesis, experiments were carried out in the presence of the protein synthesis inhibitor, cycloheximide. Exposure of EMT-6 or MGH U1 cells in the presence of up to 10 μg ml⁻¹ of cycloheximide for 18 h did not result in any decrease in clonogenic survival at either pH₆ 6.6 or 7.4 although growth of the cultures was inhibited (data not shown). After 18 h incubation at pH₆ 6.6 in the presence of cycloheximide (3 μg ml⁻¹) there was no increase in the combined activity of the Na⁺/H⁺ exchanger and the Na⁺-dependent HCO₃⁻ exchanger in MGH U1 cells when compared to controls incubated at pH₆ 7.4 in the absence of drug (Figure 1). This result is not influenced by possible differences in the number of cells in the presence or absence of cycloheximide, since pH₆ was determined as a ratio of fluorescence at pH-sensitive and insensitive wavelengths. The result implies that increased capacity for regulation of pH₆ after chronic exposure to acidic conditions is dependent on protein synthesis. Similar results were obtained for EMT-6 cells (data not shown).

We also evaluated the time taken for the activity of the exchangers to return to normal after cells were placed in pH₆ 7.4 medium following an 18 h incubation at pH₆ 6.6. In MGH U1 cells, the combined activity of the Na⁺/H⁺ antiport and the Na⁺-dependent HCO₃⁻/Cl⁻ exchanger returned to control values over 8 h (Figure 2).

Potential of flow cytometry to measure pH₆ in subpopulations of spheroids and tumours

A flow-cytometric method for the measurement of pH₆ in cells derived from dissociated tumours has been described (Hedley & Jorgensen, 1989). This technique is based on dissociation of the tumour into cold, bicarbonate-free buffer, containing amiloride, in order to prevent changes in pH₆ during preparation of a single cell suspension for flow cytometry, any change in the level of pH₆ during processing of the sample would lead to inaccuracy of the measured values.

In order to adapt this method for the measurement of pH₆ in cells from subpopulations of spheroids and tumours, we first modelled the changes in pH₆ that take place during sample preparation. Experiments were carried out at room temperature because of condensation on the cuvette at 4°C. There was considerable drift of the level of pH₆ in MGH U1 cells suspended in NMG buffer containing EIPA. This drift

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Table 1 Rates of H⁺ efflux (in nm H⁺/min) in EMT-6 and MGH U1 cells after 18 h incubation at the level of pH₆ indicated. Results have been corrected for differences in buffering capacity, and are the mean ± s.e.m. of at least three experiments.

|            | Incubation pH |
|------------|--------------|
| EMT-6      | pH₆ 7.4      |
| Na⁺/H⁺ exchanger | 3.2 ± 0.1 | 4.7 ± 0.2 |
| Na⁺-dependent Cl⁻/HCO₃⁻ exchanger | 2.0 ± 0.2 | 3.1 ± 0.2 |
| Both combined | 4.9 ± 0.5 | 7.7 ± 1.3 |
| MGH U1     | pH₆ 6.6      |
| Na⁺/H⁺ exchanger | 5.8 ± 0.8 | 8.9 ± 0.4 |
| Na⁺-dependent Cl⁻/HCO₃⁻ exchanger | 6.4 ± 0.3 | 9.4 ± 0.4 |
| Both combined | 12.0 ± 0.8 | 19.4 ± 0.2 |

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Figure 1 Combined rate of H⁺ efflux due to activity of the Na⁺/H⁺ exchanger and the Na⁺-dependent Cl⁻/HCO₃⁻ exchanger in MGH U1 cells after incubation at the pH indicated for 1 (diamonds), 6 (triangles) or 18 (open squares) hours. Rates are also shown for 18 h exposure in the presence of 3 μg ml⁻¹ cycloheximide (solid squares). Results have been corrected for differences in buffering capacity and are expressed as a percentage of the rate in control cells incubated at pH 7.4. Each point represents the mean of at least three experiments. Bars, s.e.m.
was maximal in the first 15–20 min. The pH, of cells which had an initial pH, of 6.6 or 7.2, drifted such that it approached a common value (Figure 3) which was dependent on the level of pH,. Similar results were obtained with EMT-6 cells. Since preparation of tumour and spheroid samples for flow cytometric measurement of pH, takes ~40–50 min, this method could not be used to measure accurately pH,. How-

ever, the changes that we observed in regulatory mechanisms (Figures 1 and 2) take place over hours, and flow cytometry could be used to study them.

Regulation of pH, in different regions of spheroids

Our previous studies have identified the Na+/H+ exchanger as the major mechanism responsible for the regulation of pH, under the microenvironmental conditions that may exist in solid tumours (Boyer & Tannock, 1992). We evaluated therefore the operation of this exchanger in cells derived from different regions of multicellular tumour spheroids grown from either MGH U1 or EMT-6 cells.

Initially we optimised the staining conditions in order to maximise the ratio of fluorescence between cells derived from the periphery and those from the centre of the spheroid. This was achieved by exposing spheroids of diameter 500–600 µm to 1.0 µM (EMT-6) or 0.5 µM (MGH U1) Hoechst 33342 for 20 min. Under these conditions, for EMT-6 cells, the fluorescence of the brightest 25% of cells was typically 8–20-fold greater than that of the dimmest 25% of cells. The corresponding difference for MGH U1 spheroids was 15- to 25-fold.

Activity of the Na+/H+ exchanger was determined initially in EMT-6 cells derived from different regions of spheroids grown in medium at pH 7.3. Cells from the centre of the spheroid had a slightly higher apparent rate of Na+/H+ exchange activity, than cells from the periphery or intermediate zone. Apparent rates of activity of membrane-based ion exchangers depend however on buffering capacity of the cells. We measured buffering capacity, therefore in cells from the different regions of the spheroid and found values to be a little higher in cells derived from the periphery of the spheroid (Table II). When the rates of activity of the Na+/H+ exchanger were corrected for buffering capacity, there was no significant difference between cells from the three regions of the spheroid (Figure 4a).

Since the pH, at the centre of EMT-6 spheroids has been reported to be 0.3–0.4 pH units lower than that of the medium (Carlsson & Acker, 1988), the conditions described above may not have resulted in levels of pH, that were low enough to cause changes in the activity of the exchanger. We therefore repeated the experiment with spheroids that were grown in medium at pH 6.6 for 18 h. In these experiments, the rate of activity of the Na+/H+ exchanger in cells derived from the centre of the spheroid was significantly greater than that of cells from the periphery (P = 0.03), even when corrected for buffering capacity (Table II and Figure 4a).

Different results were obtained for cells derived from MGH U1 spheroids. In these spheroids, the activity of the Na+/H+ exchanger (corrected for buffering capacity) was greater in cells derived from the centre of the spheroid even when grown at pH, 7.4 (P = < 0.01 for central vs peripheral cells) (Table II and Figure 4b). The activity of the Na+/H+ exchanger in cells from all regions of these spheroids was enhanced 1.3 to 1.5-fold following 18 h growth in medium with pH 6.6 (Figure 4b) and the differences between cells from the periphery and centre remained significant (P = 0.01).

Table II Buffering capacity of cells derived from different regions of spheroids after growth for 18 h in medium at pH 6.6 or 7.4. Results are the mean ± s.e.m. of at least four experiments

| Cell location | pH, 7.4  | pH, 6.6 |
|---------------|----------|---------|
| EMT-6         |          |         |
| Periphery     | 25.4 ± 6.3 | 21.4 ± 7.0 |
| Intermediate  | 22.9 ± 3.6 | 20.5 ± 4.0 |
| Centre        | 21.4 ± 3.5 | 19.2 ± 5.2 |
| MGH U1        |          |         |
| Periphery     | 33.6 ± 2.2 | 31.6 ± 1.0 |
| Intermediate  | 36.8 ± 1.9 | 34.1 ± 0.8 |
| Centre        | 32.4 ± 3.9 | 33.5 ± 1.0 |
pH of tumours

Values of pH were measured in EMT-6 tumours grown in Balb/c BYJ mice and in MGH U1 tumours in Swiss nude mice. In EMT-6 tumours the pH was 7.49 ± 0.04 (mean ± s.e.m. of 20 measurements in five tumours). This was significantly lower than the pH of normal muscle which was 7.49 ± 0.04 (mean ± s.e.m. of 20 measurements in five animals, P < 0.01). For MGH U1 tumours, the corresponding value was 7.11 ± 0.03 with normal muscle having a mean pH of 7.46 ± 0.02 (P < 0.01). Thus both the tumours studied had significant extracellular acidity.

Na\(^{+}\)/H\(^{+}\) exchange activity in cells from tumours

The use of Hoechst 33342 in vivo allowed the isolation of cells from different regions of tumours, based on their proximity to the blood supply. In order to demonstrate that we were able to obtain cells from hypoxic regions of tumours we measured cell survival after irradiation. Following 15 Gy delivered as a single dose, the survival of MGH U1 cells staining dimly with Hoechst 33342 (i.e. cells furthest from the blood supply) was approximately 10-fold greater than that of cells which stained brightly (data not shown). In these tumours, the mean fluorescence intensity of the brightest 25% of cells was ten times greater than that of the dimmest 25% of cells.

We next carried out experiments to assess the effect of location of tumours on the operation of the Na\(^{+}\)/H\(^{+}\) exchanger. In cells derived from EMT-6 tumours grown in Balb/c BYJ mice, a 15-fold gradient was obtained between the brightest and dimmest 25% of cells. For EMT-6 tumours, there was a small, non-significant difference in the rate of activity of the Na\(^{+}\)/H\(^{+}\) exchanger (corrected for buffering capacity) in cells from different regions, with the cells furthest from the blood supply having the lowest rate of activity (Table III). A similar pattern was observed in experiments performed with MGH U1 cells grown in nude mice (Table III).

Discussion

We have carried out experiments which assess the influence of chronic exposure of cells to reduced levels of pH, on the operation of the Na\(^{+}\)/H\(^{+}\) exchanger and the Na\(^{-}\)-dependent Cl\(^{-}\}/HCO\(_3\)^{−} exchanger. Our results indicate that in monolayer culture a reduced level of pH results in enhanced activity of both of these exchangers. In spheroids and in vivo only the Na\(^{+}\)/H\(^{+}\) exchanger was assessed; in subpopulations that are known or expected to exist in an acidic microenvironment the activity of this exchanger is increased in spheroids but not in cells derived from tumours grown in vivo.

The experiments carried out with cells growing in monolayer revealed a 1.6-fold increase in the activity of both the Na\(^{+}\)/H\(^{+}\) exchanger and the Na\(^{-}\)-dependent Cl\(^{-}\}/HCO\(_3\)^{−} exchanger. The enhancement of activity occurred after incubation at pH 6.6 for 18 h, and was prevented by cycloheximide, an inhibitor of protein synthesis. Higher levels of pH, or shorter periods of exposure resulted in smaller increases in the activity of the exchangers. These results suggest that the observed increase in activity is due either to synthesis of new exchangers, or to the synthesis of a regulatory protein. Our results agree with those obtained for cultured renal proximal tubular cells although in these cells, increased activity was noted at levels of pH as high as a 7.1 following 48 h incubation (Horie et al., 1990, 1992). The increase in the activity of the Na\(^{+}\)/H\(^{+}\) exchanger was associated with increased abundance of Na\(^{+}\)/H\(^{+}\) antiport mRNA, suggesting that the enhanced activity was due to the synthesis of new exchangers rather than a regulatory protein (Moe et al., 1991). The effect of chronic acidosis in vivo on the operation of HCO\(_3\)^{−} exchangers in membrane vesicles derived from rat renal tubular cells has also been assessed (Grassl, 1991). Chronic acidosis causes an increase in the activity of several HCO\(_3\)^{−} exchangers, although the Na\(^{-}\)-dependent Cl\(^{-}\}/HCO\(_3\)^{−} exchanger was not studied specifically. The molecular basis for the increase in HCO\(_3\)^{−} transport has not been defined.

We also examined whether the range of microenvironmental conditions encountered in multicellular tumour spheroids influenced the operation of the Na\(^{+}\)/H\(^{+}\) exchanger. In addition to reduced levels of pH, cells growing near the central regions of spheroids may be subject to hypoxia, and increased concentrations of catabolites (Carlsson & Acker, 1988); cells in this environment have a low rate of cell proliferation and may have a decreased rate of protein synthesis. Our results indicate that cells derived from the central regions of spheroids tend to have slightly higher activity of their Na\(^{+}\)/H\(^{+}\) exchangers than those from the periphery. In MGH U1 tumour this effect was observed in medium at pH 7.4, while in EMT-6 spheroids, a reduction in the level of the pH of the medium was necessary to observe this effect. Surprisingly, in EMT-6 spheroids, the activity of the Na\(^{+}\)/H\(^{+}\) exchanger in peripheral cells was no higher when the spheroids had been grown in pH 6.6 medium than when they were grown at pH 7.4. The explanation for this finding is not

![Figure 4](https://via.placeholder.com/150)

Figure 4 Rate of H\(^{+}\) efflux due to activity of the Na\(^{+}\)/H\(^{+}\) exchanger in cells derived from different regions of EMT-6 a and MGH U1 b, spheroids following 18 h of growth in medium at pH 7.4 (open bars) or 6.6 (solid bars). Results have been corrected for differences in buffering capacity, and are the means ± s.e.m. of at least six experiments.

**Table III** Rate of H\(^{+}\) efflux (in mm H\(^{-}\)/min) due to activity of the Na\(^{+}\)/H\(^{+}\) exchanger in cells derived from different regions of EMT-6 and MGH U1 tumours. Results have been corrected for buffering capacity and are the mean ± s.e.m. from at least seven tumours.

| Tumour     | Relationship to functional blood vessel |
|------------|----------------------------------------|
|            | Closest      | Intermediate | Farthest     |
| EMT-6      | 4.5 ± 0.4  | 4.6 ± 0.2   | 4.1 ± 0.3   |
| MGH U1     | 7.8 ± 0.1  | 7.6 ± 0.2   | 7.0 ± 0.9   |
clear, but it is possible that when this cell line is grown as a sphere, lower levels of pH₄ are necessary to stimulate overexpression of the Na⁺/H⁺ exchanger. The different patterns of enhancement of Na⁺/H⁺ antiport activity observed in the two sphereoid systems may be due to differences in microenvironmental conditions. Data are available for EMT-6 spheroids concerning the levels of pH₄ and pO₂ at different depths (Carlsson & Acker, 1988), but different sublines are likely to show genetic drift, and these results might not be directly applicable to EMT-6 spheroids grown in our laboratory. There are no data relating to the distribution of pH₄ and pO₂ in MGH U1 spheroids. Spheroids derived from different cell lines are known to grow at different density (cells/volume of spheroid), which could result in important differences in the microenvironment. We fail to detect any significant difference in the rate of activity of the Na⁺/H⁺ exchanger in cells from tumours based on their proximity to the functional blood supply. There are several possible explanations for this finding, and for the apparent disparity with our in vitro observations. Measurements of pH₄ in EMT-6 tumours revealed a mean ± s.e.m. value of 6.91 ± 0.05. Although some regions within the tumour could be expected to have levels of pH₄ lower than the mean, the local values of pH₄ may not be low enough to cause Na⁺/H⁺ antiport activity of the Na⁺/H⁺ exchanger. In MGH U1 tumours, the level of pH₄ was even higher and this may account for the lack of enhancement of Na⁺/H⁺ exchange activity in cells from these tumours. Within solid tumours, both acute (perfusion limited) and chronic (diffusion limited) hypoxia occur (Chaplin et al., 1989; Minchinton et al., 1990). It is probable that cells from regions subject to acute interruptions in blood flow are not exposed to low levels of pH₄ for a sufficient length of time to cause upregulation of the Na⁺/H⁺ exchanger. Although cells from areas of chronic hypoxia may have a sufficient duration of exposure to low levels of pH₄ to cause upregulation of exchange activity, the viable cells from these regions comprise a small proportion of the whole tumour. The flow cytometric method allows separation of cells only into quite large subpopulations, and there will be some contamination with cells from neighbouring regions of tumours. If severely acidic cells comprised a small population (<10%), upregulation of Na⁺/H⁺ exchange might not be detected in our experiments. Finally, there are differences between in vitro and in vivo experiments that cannot be controlled for. In the monolayer experiments, the only factor modified was the level of pH₄. By contrast, cells growing in a tumour are exposed not only to reduced levels of pH₄ but also to hypoxia, and deprivation of other nutrients. These conditions may combine to inhibit energy metabolism or protein synthesis, and prevent up-regulation of Na⁺/H⁺ exchange. Furthermore, the presence of growth factors and the products of host cells that infiltrate the tumour could modulate the response of cells to microenvironmental conditions.

Agents which interfere with the ability of cells to regulate pH₄ have been proposed as potential anticancer agents (Tannock & Rotin, 1989; Newell et al., 1992; Maidorn et al., 1993). Furthermore, the cytotoxic effects of hyperthermia are enhanced by a reduction in the level of pH₄ (Chu et al., 1990; Lyons et al., 1992). The success of strategies such as these depends on an understanding of how pH₄ is regulated within tumours in vivo, and what factors modulate this regulation. We have shown previously that under the acidic conditions that are likely to exist within solid tumours, the Na⁺/H⁺ exchanger is the major mechanism that is responsible for the regulation of pH₄ (Boyer & Tannock, 1992). Our finding that cells in vivo do not up-regulate their Na⁺/H⁺ exchanger implies that these cells are unlikely to be more resistant to the effects of drugs targeted against this antiporter than cells in a less acidic environment. We conclude therefore, that the Na⁺/H⁺ exchanger remains an appropriate target for anticancer therapy.

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