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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 (pH_i). We have therefore developed a method, based on dual staining of cells and flow cytometry, to study the regulation of pH_i in subpopulations of tumours and spheroids. The activity of membrane-based pH_i regulating transporters was studied in EMT-6 and MGH U1 cells grown in monolayer culture, spheroids, and tumours. pH_i 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_e 6.6 led to a 1.3-1.5-fold enhancement in the activity of both the Na^+/H^+ exchanger and the Na^+-dependent $Cl^-//HCO_3^-$ 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 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 (pH_e) 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 pH_e within solid tumours is approximately 0.5 pH units lower than that in normal tissues, techniques that measure predominantly intracellular pH (pH_i), such as ^{31}P nuclear magnetic resonance spectroscopy, suggest similar values of pH_i in tumours and normal tissues (Vaupel *et al.*, 1989). These observations imply that tumours cells are able to regulate pH_i under the acidic conditions encountered within solid tumours.

Three major mechanisms allow cells to regulate their pH_i 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^+-dependent Cl^-/HCO_3^- exchanger. The buffering capacity of a cell is its ability to buffer a change in pH_i 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 pH_i is the Na^+-dependent HCO_3^-/Cl^- exchanger. This exchanger has been detected in most but not all cell lines tested (Reinertsen *et al.*, 1988; Tonnessen *et al.*, 1990). It employs the inwardly directed Na^+ gradient to exchange intracellular Cl^- for extracellular HCO_3^-, and is inhibited by the stilbene derivative 4-4'-diisothiocyanostilbene-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 HCO_3^- exchangers, the mechanisms have not been characterised (Grassl, 1991).

Previous studies in tissue culture have suggestged 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 pH_i (Boyer & Tannock, 1992). Agents that inhibit the operation of this exchanger show considerable potential for causing pH_e-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 pH_i 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 pH_i 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 α 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). Hoechst 33342 was obtained from Aldrich (Milwaukee, WI, USA). DIDS was purchased from ICN Biomedicals (St Laurent, PQ, Canada). 2'7'-bis-(2-carboxyethyl)-5-(and-6)carboxyfluorescein (BCECF) acetoxymethyl 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_i and its regulation for cells in monolayer

Cells grown as a monolayer on glass coverslips were exposed to 2 μg ml^{-1} of the acetoxymethyl 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_i 6.0–7.5, fluorescence intensity of BCECF at 525 nM (following excitation at 495 nM) is linearly related to pH_i (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_i, and depends only on the amount of BCECF present. The ratio of fluorescence at pH-dependent and independent wavelengths provides therefore an estimate of pH_i 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_4Cl for 30 min. Acidification to a level determined by the concentration of NH_4^+ used, was then produced by exchanging the NH_4Cl containing solution with a NH_4Cl-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 alkalinisation was measured by the fluorometre. The results of these experiments were converted into H^+ efflux by multiplying the observed rates of change of pH_i 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_e on the activity of the pH_i 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-based exchangers which regulate pH_i. 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_i 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 i.e. $\Delta[H^+]/\Delta pH_i$ (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_i 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_i only, since values have been shown to be constant over the range of pH_i 6.4–7.2 (Grinstein *et al.*, 1984). Bicarbonate buffering capacity was calculated as 2.3 $[HCO_3^-]_i$ (Boron, 1989). The value of $[HCO_3^-]_i$ was calculated from knowledge of pH_i, pH_e and $[HCO_3^-]_e$. 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 (Erlichman & 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.

Subpopulations of cells at different depths in spheroids were obtained by exposure 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 disaggregation.

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 $2.5-5 \times 10^5$ 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 the 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 pH_i from dissociated spheroids and tumours

We wished to adapt a flow cytometric method to measure pH_i 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 pH_i 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 pH_i to either 6.6 or 7.2, the cells were then centrifuged and resuspended in KCl solution at pH_e 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 pH_e; this solution provides complete inhibition of Na^+/H^+ and Na^+-dependent HCO_3^-/Cl^- exchangers that regulate pH_i under acidic conditions. The cell suspensions were then placed into two cuvettes in an Aminco-Bowman Series 2 Fluorometer (SLM, Urbana, IL, USA); this machine allows rapid alternation of fluorescence measurements from two cuvettes and pH_i was monitored, using the ratio method described earlier, by alternating between each of the cuvettes for up to 60 min.

Evaluation of pH_i and its regulation in dissociated spheroids and tumours

Regulation of pH_i 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 pH_i 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 μg 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 NH_4^+ (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 han measuring the fall in pH_i following removal of NH_4^+, the immediate increase in pH_i resulting from exposure of cells to 5 mM NH_4^+ was determined. The measurements and calculations were otherwise identical.

Regulation of pH_i 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 pH_i after addition of sodium was then determined for each of these subpopulations, by calculating the average pH_i 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 pH_i. 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 pH_i. 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, unanesthetised and unrestrained tumour bearing mice were irradiated with a dose of 15 Gy using a ^{137}Cs 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 pH_i regulation experiments described above. The sorted cells were incubated in culture dishes containing α-medium, in a humidified atmosphere containing 5% CO_2 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_e in vivo

Mice were anesthetised with sodium pentobarbital (M.T.C. Pharmaceuticals; Cambridge, Ontario), 65 mg kg^{-1} body weight. Measurements of pH_e 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 pH_e 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_e exposure on regulation of pH_i

Initial experiments were undertaken to determine whether the activity of the Na^+/H^+ exchanger and the Na^+-dependent Cl^-/HCO_3^- exchanger were altered by growth of cells under acidic conditions. Cells were grown for up to 18 h at levels of pH_e in the range 6.6 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 pH_e dependent increase in the combined activity of the Na^+/H^+ exchanger and the Na^+-dependent Cl^-/HCO_3^- exchanger in MGH U1 cells (Figure 1). The maximum increase, a 1.6-fold enhancement of activity, occurred after 18 h incubation at pH_e 6.6; evaluation after longer times was not undertaken because of loss of cellular viability. Similar results were obtained with EMT-6 cells (Table I).

The apparent increase in the combined activity of the Na^+/H^+ antiport and the Na^+-dependent Cl^-/HCO_3^- exchanger could have been due to changes in the intrinsic buffer capacity of the cell after low pH_e exposure. We therefore measured intrinsic buffering capacity after MGH U1 cells had been growing at pH_e 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

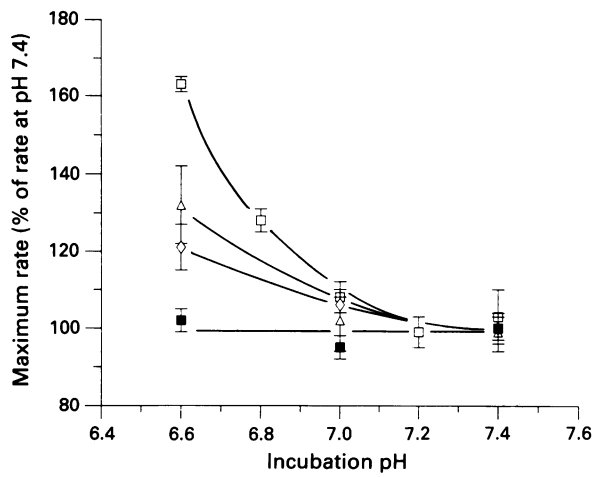

Figure 1 Combined rate of H^+ efflux due to activity of the Na^+/H^+ exchanger and the Na^+-dependent Cl^-/HCO_3^- exchanger in MGH U1 cells after incubation at the pH indicated for 1 (diamonds), 6 (triangles) or 18 (open squares) hours. Rates are also shown for 18 h exposure in the presence of 3 μg 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.

Table I Rates of H^+ efflux (in mM H^+/min) in EMT-6 and MGH U1 cells after 18 h incubation at the level of pH_e 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	*pH_e 7.4*	*pH_e 6.6*
Na^+/H^+ exchanger	3.2 ± 0.1	4.7 ± 0.2
Na^+-dependent Cl^-/HCO_3^- exchanger	2.0 ± 0.2	3.1 ± 0.2
Both combined	4.9 ± 0.5	7.7 ± 1.3
MGH U1		
Na^+/H^+ exchanger	5.8 ± 0.8	8.9 ± 0.4
Na^+-dependent Cl^-/HCO_3^- exchanger	6.4 ± 0.3	9.4 ± 0.4
Both combined	12.0 ± 0.8	19.4 ± 0.2

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^-/HCO_3^- 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 HCO_3^- (to measure activity of the Na^+/H^+ exchanger) or in the presence of EIPA (to measure activity of the Na^+-dependent Cl^-/HCO_3^- exchanger). Table I shows that the rate of pH_i 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 pH_e 6.6 or 7.4 although growth of the cultures was inhibited (data not shown). After 18 h incubation at pH_e 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 HCO_3^- exchanger in MGH U1 cells when compared to controls incubated at pH_e 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 pH_i was determined as a ratio of fluorescence at pH-sensitive and insensitive wavelengths. The result implies that increased capacity for regulation of pH_i 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 pH_e 7.4 medium following an 18 h incubation at pH_e 6.6. In MGH U1 cells, the combined activity of the Na^+/H^+ antiport and the Na^+-dependent HCO_3^-/Cl^- exchanger returned to control values over 8 h (Figure 2).

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

A flow-cytometric method for the measurement of pH_i 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 pH_i during preparation of a single cell suspension for flow cytometry; any change in the level of pH_i during processing of the sample would lead to inaccuracy of the measured values.

In order to adapt this method for the measurement of pH_i in cells from subpopulations of spheroids and tumours, we first modelled the changes in pH_i 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 pH_i in MGH U1 cells suspended in NMG buffer containing EIPA. This drift

was maximal in the first 15–20 min. The pH_i of cells which had an initial pH_i of 6.6 or 7.2, drifted such that it approached a common value (Figure 3) which was dependent on the level of pH_e. Similar results were obtained with EMT-6 cells. Since preparation of tumour and spheroid samples for flow cytometric measurement of pH_i takes ~40–50 min, this method could not be used to measure accurately pH_i. However, 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.

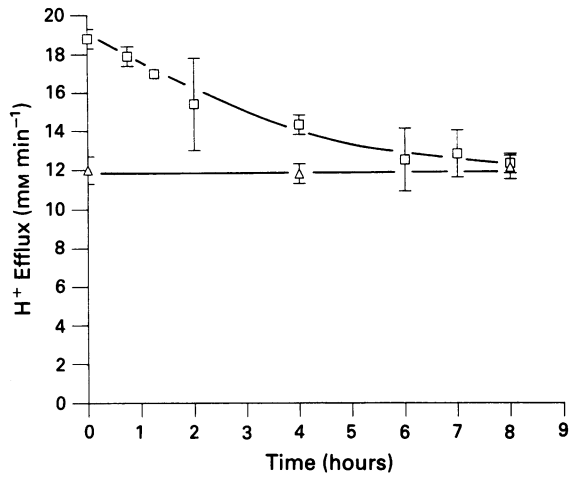

Figure 2 Combined rate of H^+ efflux due to activity of the Na^+/H^+ exchanger and the Na^+-dependent Cl^-/HCO_3^- exchanger in MGH U1 cells at varying lengths of time following removal from pH 6.6 medium, in which cells had been incubated for 18 h (squares). Triangles indicate cells incubated in medium with pH_e 7.4. Each point is the mean of at least two experiments. Bars, s.e.m.

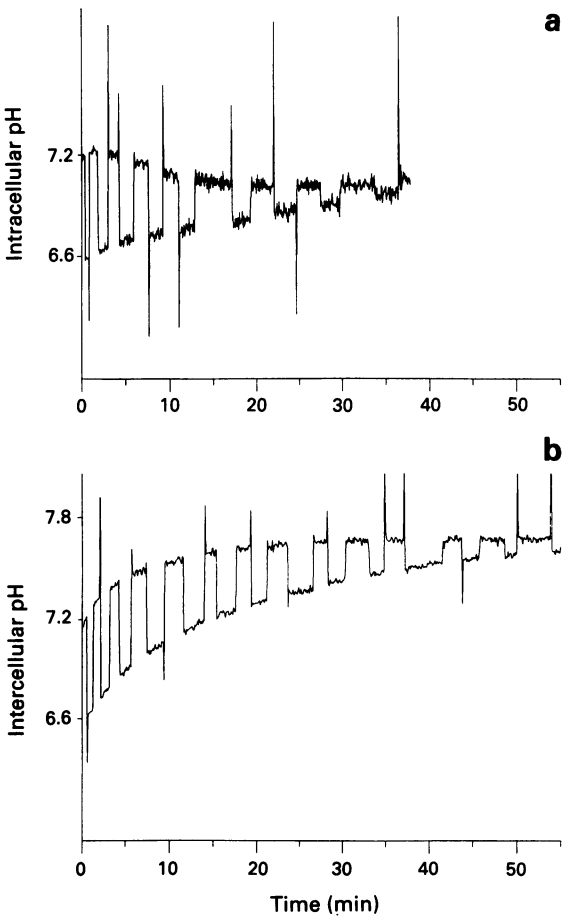

Figure 3 The pH_i of MGH U1 cells was brought to an initial value of either 6.6 or 7.2 by suspension in KCl solution in the presence of nigericin. The cells were then placed in solution designed to inhibit regulation of pH_i (NMG solution containing EIPA) and change in pH_i was monitored at **a**, pH_e 7.0 and **b**, pH_e 7.8.

Regulation of pH_i in different regions of spheroids

Our previous studies have identified the Na^+/H^+ exchanger as the major mechanism responsible for the regulation of pH_i 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 conditons, 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_e 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_e 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_e 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_e 7.4*	*pH_e 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

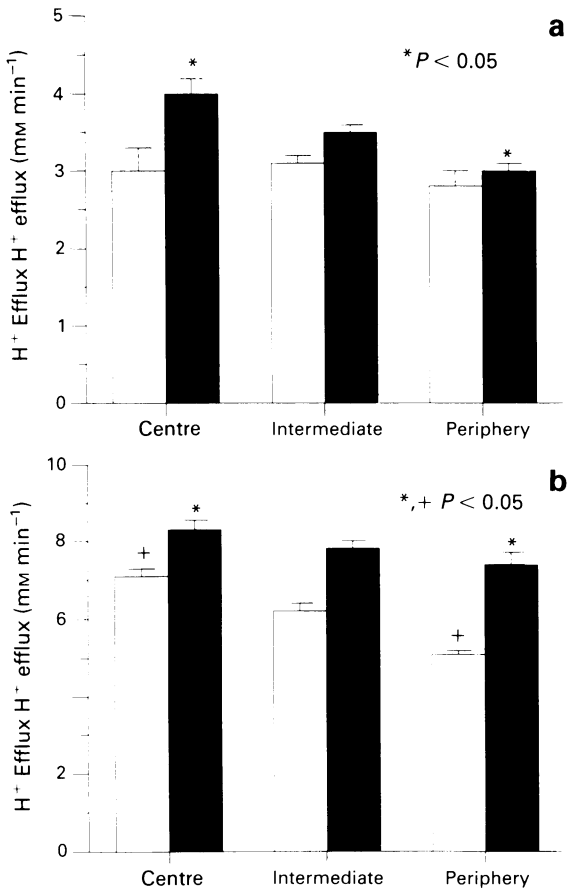

Figure 4 Rate of H^+ efflux due to activity of the Na^+/H^+ exchanger in cells derived from different regions of EMT-6 **a**, and MGH U1 **b**, spheroids following 18 h of growth in medium at pH 7.4 (open bars) or 6.6 (solid bars). Results have been corrected for differences in buffering capacity, and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

pH_e of tumours

Values of pH_e 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_e was 6.91 ± 0.05 (mean ± s.e.m. of 20 measurements in five tumours). This was significantly lower than the pH_e 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_e 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 cells 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_e 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_e 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_e 6.6 for 18 h, and was prevented by cycloheximide, an inhibitor of protein synthesis. Higher levels of pH_e, 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_e 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_e, 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 spheroids 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_e 6.6 medium than when they were grown at pH_e 7.4. The explanation for this finding is not

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

	Relationship to functional blood vessel		
Tumour	*Closest*	*Intermediate*	*Furthest*
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 spheroid, lower levels of pH_e are necessary to stimulate overexpression of the Na^+/H^+ exchanger.

The different patterns of enhancement of Na^+/H^+ antiport activity observed in the two spheroid systems may be due to differences in microenvironmental conditions. Data are available for EMT-6 spheroids concerning the levels of pH_e and pO_2 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 pH_e and pO_2 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 failed 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 pH_e 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 pH_e lower than the mean, the local values of pH_e may not be low enough to cause enhanced activity of the Na^+/H^+ exchanger. In MGH U1 tumours, the level of pH_e 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 pH_e 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 pH_e 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 pH_e. By contrast, cells growing in a tumour are exposed not only to reduced levels of pH_e 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 pH_i 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 pH_i (Chu *et al.*, 1990; Lyons *et al.*, 1992). The success of strategies such as these depends on an understanding of how pH_i is regulated within tumours *in vivo*, and what factors modulate this regulation. We have shown previously that under the acidic conditons that are likely to exist within solid tumours, the Na^+/H^+ exchanger is the major mechanism that is responsible for the regulation of pH_i (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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