ELSEVIER

Biochimica et Biophysica Acta 1282 (1996) 131–139

BBA Biochimica et Biophysica Acta

Effects of extracellular pH on intracellular pH-regulation and growth in a human colon carcinoma cell-line

Georg Bischof [a,*], Enrico Cosentini [a], Gerhard Hamilton [a], Martin Riegler [a], Johannes Zacherl [a], Bela Teleky [a], Wolfgang Feil [a], Rudolf Schiessel [a], Terry E. Machen [b], Etienne Wenzl [a]

[a] *University Clinic of Surgery, Währingergürtel 18–20, A-1090 Vienna, Austria*

[b] *Department of Molecular and Cell Biology, 231 / 235 Life Sciences Addition, University of California, Berkeley, CA 94720, USA*

Received 13 July 1995; accepted 1 March 1996

Abstract

Mechanisms of intracellular pH (pH_i) regulation seem to be involved in cellular growth and cell division. Little is known about how extracellular acidosis, known to occur in central regions of solid tumors, or alkaline conditions affect pH_i regulation in colonic tumors. pH_i changes in the colonic adenocarcinoma cell-line SW-620 were recorded by spectrofluorimetric monitoring of the pH-sensitive, fluorescent dye BCECF, and proliferative activity was assessed by [^{3}H]thymidine uptake. Resting pH_i in Hepes-buffered solution was 7.53 ± 0.01 ($n = 36$). Both 1 mM amiloride and Na^+-free solution inhibited pH_i recovery from acidification and decreased pH_i in resting cells. In HCO_3^-/CO_2-buffered media resting pH_i was 7.42 ± 0.01 ($n = 36$). Recovery from acidification was Na^+-dependent, Cl^--independent, and only partially blocked by 1 mM amiloride. In the presence of amiloride and 200 μM H_2DIDS pH_i recovery was completely inhibited. In Na^+-free solution pH_i decreased from 7.44 ± 0.04 to 7.29 ± 0.03 ($n = 6$) and no alkalinization was observed in Cl^--free medium. Addition of 5 μM tributyltin bromide (an anion/OH^- exchange ionophore) caused pH_i to decrease from 7.43 ± 0.05 to 7.17 ± 0.08 ($n = 5$). The effects of pH_o on steady-state pH_i, pH_i recovery from acidification and proliferative activity after 48 h were investigated by changing buffer [CO_2] and [HCO_3^-]. In general, increases in pH_o between 6.7 and 7.4 increased pH_i recovery, steady-state pH_i and growth rates. In summary, SW-620 cells have a resting $pH_i > 7.4$ at 25°C, which is higher than other intestinal cells. Acid extrusion in physiological bicarbonate media is accomplished by a pH_i-sensitive Na^+/H^+ exchanger and a pH_i-insensitive Na^+-HCO_3^- cotransporter, both of which are operational in control cells at the resting pH_i. No evidence for activity of a Cl^-/HCO_3^- exchanger was found in these cells, which could account for the high pH_i observed and may explain why the cells continue to grow in acidic tumor environments.

Keywords: pH, intracellular; pH regulation; Growth; Carcinoma cell; (Human colon)

1. Introduction

Carcinoma cells are often resistant to radiation and chemotherapy and therefore represent a major target for experimental and clinical studies dealing with new anti-tumor strategies. The maintenance of intracellular pH (pH_i) within a narrow range is of vital importance for each cell, as most cellular functions are pH-sensitive [18]. The Na^+/H^+ exchanger has been found to counteract cytoplasmic acidification and to regulate cell volume and intracellular Na^+ concentration [11]. Furthermore, this mechanism plays an important role in the onset of cellular growth, as it is directly activated by growth factors and tumor promoting agents (phorbol esters, diacylglycerol) [5,15,16].

It has also been demonstrated that the Na^+/H^+ exchanger is required for tumor growth [25]. Thus, Na^+/H^+ antiport-deficient subpopulations of human bladder carcinoma cells were no longer able to form spheroids or induce tumor growth in-vivo, and revertant cells with tumorigenic capacity regained the Na^+/H^+ exchanger activity. In addition the tumor cells were selectively killed by reducing pH_i [17], which was achieved by reducing extracellular pH (pH_o) below 7.3 during simultaneous

* Corresponding author. Fax: +43 1 404005641; e-mail: georg.bischof@vm.akh-wien.ac.at.

PII S0005-2736(96)00050-8

application of the proton ionophore CCCP (carbonyl cyanide 3-chlorophenylhydrazone) along with amiloride and DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid). These agents are known to inhibit membrane-based pH_i regulating mechanisms and the findings could be of great interest for treatment of solid tumors, which often exhibit central acidic regions [26].

Mechanisms of pH_i regulation in human colonic epithelial cells, which are exposed to considerable proton gradients, are a current topic of ongoing studies. We have recently demonstrated roles for Na^+/H^+ exchange, Na^+-HCO_3^- cotransport and Na^+-dependent Cl^-/HCO_3^- exchange in freshly isolated human colonic crypt cells [29]. Several studies have suggested an effect of pH_o on steady-state pH_i [6,21,28], and systemic acid-base disorders have been found to change colonic mucosal pH_i and ion transport in rats [34], mediated by changes in the intracellular acid-base milieu.

In order to gain insight in how tumor cells of the large intestine are affected by altered environmental conditions, we studied pH_i regulatory mechanisms of SW-620 cells, a cell-line derived from a human colonic adenocarcinoma [13], both in Hepes- as well as in bicarbonate-buffered media. We found that these tumor cells had an unusually high resting pH_i at 25°C (> 7.4) compared to normal colonic epithelial cells. We therefore determined mechanisms of pH_i regulation to gain insight into the genesis of the high steady-state pH_i. We found that, like many other cells, SW-620 cells had Na^+/H^+ exchange and Na^+-HCO_3^- cotransport, but Cl^--dependent pH_i regulation (including the acid-loading Cl^-/HCO_3^- exchange) was not operational. We then tested the effects of changing pH_o on steady-state pH_i, pH_i regulation and growth to gain insight into how acidotic or alkalotic conditions, either of which may exist inside tumors, may affect these cells in their 'physiological state'.

2. Materials and methods

2.1. Tissue culture and BCECF-loading of cells

SW-620 cells originate from a lymph-node metastasis of a patient with colonic adenocarcinoma [13] and show ultrastructure typical of epithelial cells, including intracellular junction complexes and microvilli [33]. Cells were purchased from the American Type Culture Collection (Rockville, MD) and grown to confluent monolayers in a humidified incubator (5% CO_2/air, 37°C) in RPMI-1640 bicarbonate-medium (Flow, Irvine, Scotland, UK). The medium contained 10% fetal bovine serum, 4 mM glutamine and 75 μg/ml gentamicin. The cells were subsequently trypsinized (0.03% trypsin containing 0.02% EDTA, three times a week on average, ratio approximately 1:5) and seeded onto 9×55 mm rectangular plastic slides of Leighton Tubes (Costar, Cambridge, MA) 24–48 h before the experiment. Experiments were performed when the cells were still in a subconfluent state.

For dye loading the cells were exposed to 4 μM of the acetoxymethyl ester of the fluorescein-derivative BCECF (2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein) for 30 min at 25°C. After permeating the cellular membrane, the dye is cleaved to its membrane-impermeant form by non-specific intracellular esterases [31] and thus remains trapped in the cytosol. The cells were then incubated in dye-free NaCl-Ringer's solution for 20 min to allow stabilization after the dye-loading procedure.

2.2. Measurement of pH_i

The slide with the dye-loaded cells was mounted in a cuvette and placed in a Perkin-Elmer LS-5B Spectrometer, that was used to alternately expose the cells to a monochrome excitation light at 439 nm and 490 nm. Slit widths were 10 nm each. The resulting fluorescence signals were collected at an emission wavelength of 526 nm. A ratioing method, dividing the pH-sensitive intensity at 490 nm by the pH-insensitive 439 nm intensity, was used to compensate for differences in number of cells and dye-quantity [19]. Changing between the two excitation wavelengths and data acquisition (about every 4 s) was controlled by fura-2 software (modified for BCECF by E. Wenzl) running on a personal computer. Bathing solutions were changed by quickly transferring the cells into another cuvette filled with the new incubation medium. In a preliminary set of experiments background fluorescence of similar cell preparations as well as fluorescence of the plastic slides was measured, and, since values did not exceed 5–7% of total fluorescence intensity, no correction was performed in further protocols. We also measured fluorescence of the bathing solutions at the end of the experiments in order to evaluate dye-leakage, and if these values exceeded 10% of the total fluorescence intensity, the data were excluded.

2.3. Calibration of fluorescence ratios in terms of pH_i

At the end of each experiment the cells were exposed to the artificial K^+/H^+ exchanger nigericin in high $[K^+]$ solution [23]. When $[K^+]_o$ equals $[K^+]_i$, nigericin sets $[H^+]_o = [H^+]_i$ ($pH_o = pH_i$), and 490 nm/439 nm ratios were obtained for four declining $pH_o = pH_i$ values, covering the range of pH 6.5–7.8, where BCECF fluorescence intensity is known to be linearly related to pH_i. By using a least squares linear fit a regression function was determined ($R > 0.95$ for all experiments).

2.4. Determination of intracellular buffer capacity, rates of pH_i changes and of H^+-efflux

We used a technique reported by Boyarski et al. [7,8] and Wenzl et al. [36] for calculation of intrinsic buffer

capacities (β_i). β_i was derived from the equation

$$\beta_i = d[H^+]_i/dpH$$

Cells were incubated in Hepes-buffered media in order to exclude effects of HCO_3^--dependent exchange mechanisms. Then Na^+-free solution was used to block the activity of the Na^+/H^+ exchanger. When NMG^+ was replaced by decreasing concentrations of NH_4^+ (30, 20, 10, 5, 0 mM) β_i could be calculated for each according change of pH_i using the Henderson–Hasselbalch equation. Total intracellular buffer capacity (β_t) was then calculated from the equation $\beta_t = \beta_i + 2.3[HCO_3^-]_i$, where $[HCO_3^-]_i$ was determined at each pH_i by use of the Henderson–Hasselbalch equation, assuming CO_2 to be at equilibrium in the cytoplasm and the extracellular space. Fig. 1 shows the dependence of β_i on pH_i. The obtained values were slightly lower than those reported for parietal cells [36] or renal mesangial cells [7]. There was a strong increase of β_{HCO3} over β_i above $pH_i = 7.4$, similar to what was recently described for isolated rat hepatocytes [2].

In several experiments rates of change of pH_i and of transmembrane H^+-efflux after intracellular acidification were calculated for different experimental conditions. Rates of pH_i change (dpH/dt) were determined from the slopes of a linear least-squares fit of the pH_i trace passing a given pH_i value.

Net H^+-efflux rates (J^H) were calculated at a given $pH_i = pH_t$ from

$$J^H_{net,pH_t} = (dpH/dt_{pH_i})(\beta_{pH_t})$$

where β_{pHt} is the cellular buffer capacity at a given pH_t, as described by Wenzl et al. [37].

2.5. Modification of external HCO_3^-/CO_2 conditions during pH_i measurements

Cells were exposed to different environmental conditions resembling in vivo conditions, ranging from uncompensated metabolic acidosis to alkalosis, by changing from Hepes- to HCO_3^-/CO_2-buffered Ringer's. Either 35 mM, 25 mM, 15 mM or 5 mM HCO_3^- was used (gassed with either 15, 5 or 2.5% CO_2, balance vol.% O_2). These protocols generated 12 experimental groups with pH_o values ranging from 6.26 to 7.88. pH_i recovery in the different HCO_3^-/CO_2 media was monitored for 15 min. The pH of the incubation media was kept constant throughout this time before switching back to Hepes solution.

2.6. Testing effects of different HCO_3^-/CO_2 conditions on proliferative activity

Cells were grown in RPMI-1640 medium (Cat. No. F1225, without bicarbonate; Biochrome, Berlin, Germany) supplemented with 10% FCS and 4 mM glutamine. Where applicable, $NaHCO_3$ was added to final concentrations of 45, 35, 25, 15, 5 mM and 2.5 mM HCO_3^-, and solutions were preequilibrated with 5% CO_2 for 24 h. Cells were trypsinized and resuspended in the different bicarbonate-media and distributed in 96-well microtiter plates. Dilution of an initial concentration of $0.5 \cdot 10^6$ cells/ml yielded final plating cell numbers of about 25 000 cells per well. Control rates of growth were obtained using standard tissue culture medium (25 mM HCO_3^-) gassed with air/5% CO_2 as well as with 95% O_2/5% CO_2. All cultures were set up in triplicate, incubated for 48 h at 37°C and labelled with 37 kBq/well [^{3}H]thymidine for the last 18 h. Cells were collected with a cell harvester (Skatron, Lier, Norway) after trypsinization, and the filter disks (Schleicher and Schuell, Dassel, Germany) were measured by liquid scintillation counting for incorporated radioactivity (Quicksafe A, Zinser, Maidenhead, UK). Data were expressed as means of counts per min per well per 48 h ± S.E.M.

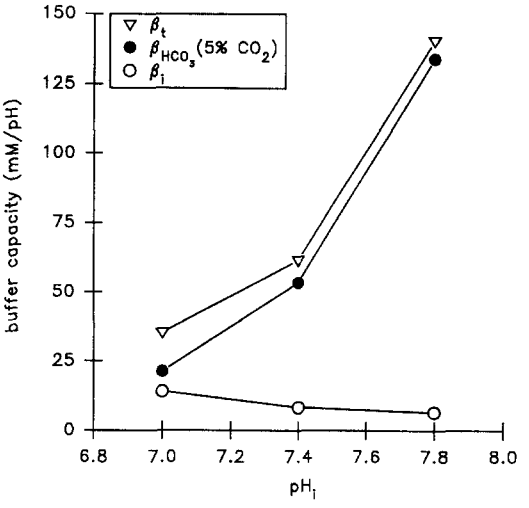

Fig. 1. Buffer capacities in SW-620 cells. Intrinsic (β_i), extrinsic (β_{HCO3}) and total buffer capacity (β_t) were determined as described in Section 2 and are shown as functions of pH_i. Values are means from 14 experiments.

2.7. Chemicals and solutions

All chemicals were from Sigma (St. Louis, MO) unless otherwise stated. 2',7'-Bis(carboxyethyl)-5(6)carboxyfluorescein-acetoxymethyl ester (BCECF/AM) and [H_2] 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (H_2DIDS) were from Molecular Probes (Eugene, OR). Tributyltin bromide was from Aldrich (Milwaukee, WI).

Hepes-buffered NaCl-Ringer's solution consisted of 150.0 mM NaCl, 2.5 mM K_2HPO_4, 1.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, 10.0 mM glucose, 20.0 mM Hepes; bicarbonate-buffered NaCl-Ringer's contained in mM: NaCl 125.0, $NaHCO_3$ 25.0, K_2HPO_4 2.5, $CaCl_2$ 1.0, $MgSO_4$ 1.0, glucose 10.0. In Na^+-free solutions Na^+ was replaced by NMG^+, in Cl^--free solutions Cl^- was replaced by gluconate or cyclamate. In NH_4Cl-containing solutions

NH_4^+ replaced Na^+ or NMG^+, in Cl^--free $(NH_4)_2SO_4$ replaced Na^+-gluconate or Na^+-cyclamate. NH_4^+ was used at a concentration of 25.0 mM, except when buffer capacities were determined.

High $[K^+]$ solutions for calibration consisted of 140.0 mM KCl, 1.3 mM $MgSO_4$, 2.0 mM $CaCl_2$, 12.0 mM glucose, 20.0 mM Hepes, and nigericin was added to a final concentration of 10 μM.

Hepes-buffered solutions were equilibrated with air, HCO_3^--buffered solutions with 5% CO_2/95% O_2 (unless otherwise stated) both in the beakers and the cuvette during measurements. pH of bathing solutions (pH_o) was 7.4 (except for calibration) and the temperature adjusted to 25°C for all measurements.

2.8. Viability of cells

Before starting experiments dye-loaded and unloaded cells of the same batch were exposed to 0.5% trypan blue. At the end of each experiment cells were checked the same way. The data were rejected when more than 10% of the cells accumulated the dye.

2.9. Statistics

Values are presented as means ± S.E.M. of six according experiments (unless otherwise stated). The Student's *t*-test for unpaired data and Analysis of Covariance was used to calculate statistical significance and $P < 0.05$ was considered significant. For comparison of proliferative activity assays Tukey's Studentized Range Test and a test for Quadratic Effects was applied.

3. Results

3.1. Steady-state pH_i in Hepes and HCO_3^-/CO_2 solutions

In Hepes-buffered NaCl-Ringer's steady-state pH_i was 7.53 ± 0.01 ($n = 36$; see Figs. 2 and 3). When Hepes-Ringer's was replaced by 25 mM HCO_3^-/5% CO_2-buffered NaCl, pH_i suddenly decreased to 7.10 ± 0.01 ($n = 36$), probably because of the quick entry of CO_2, and recovered to 7.39 ± 0.01 within 8 min (Fig. 3). The mean steady-state pH_i after a stabilization period of 24 min in HCO_3^-/CO_2-NaCl was 7.42 ± 0.01, which was 0.11 units lower than in Hepes-NaCl ($P < 0.001$).

3.2. Evidence for Na^+/H^+ exchange

A convenient method to study acid extruding mechanisms is to induce cytoplasmic acidification and to monitor subsequent pH_i recovery under different experimental conditions. Applying the well known ammonium-prepulse technique [24], SW-620 cells were pulsed with 25 mM NH_4Cl in Hepes-buffer. pH_i first rose to a value of

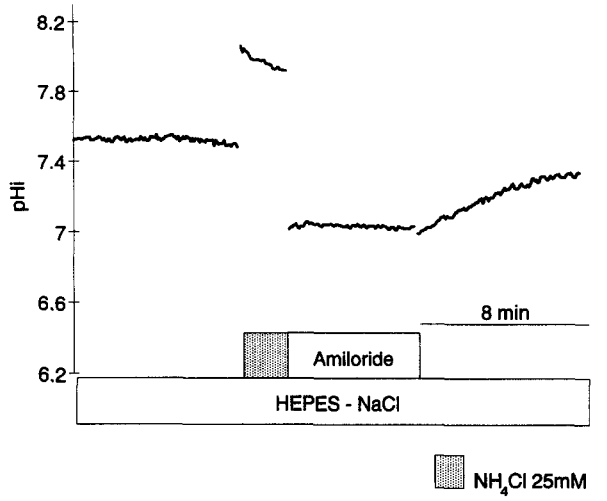

Fig. 2. Effect of 1 mM amiloride on pH_i recovery after intracellular acid-loading in Hepes-buffered solutions. Cells were acidified using the ammonium-prepulse technique with 25 mM NH_4Cl. Subsequent incubation of cells with 1 mM amiloride in Hepes-NaCl completely blocked pH_i recovery during the 8 min of exposure to amiloride. When amiloride was removed, pH_i increased towards normal values. The trace is representative of six similar experiments.

8.08 ± 0.02 due to the quick entry of NH_3 (Fig. 2). After 2 min NH_4^+ was removed from the external solution, and pH_i dropped from 7.95 ± 0.03 to 7.05 ± 0.07. pH_i recovery was completely blocked by addition of 1 mM amiloride, a known inhibitor of Na^+/H^+ exchange [3], to NaCl Ringer's. When amiloride was removed, pH_i recovered back to 7.43 ± 0.05 within 8 min. When the cells were exposed to Na^+-free medium after the NH_4^+ pulse, cells acidified to pH_i 7.06 ± 0.08 and remained acidic (7.06 ± 0.07) for 8 min (not shown).

In another set of experiments the effects of Na^+-free solution or 1 mM amiloride on resting pH_i were investi-

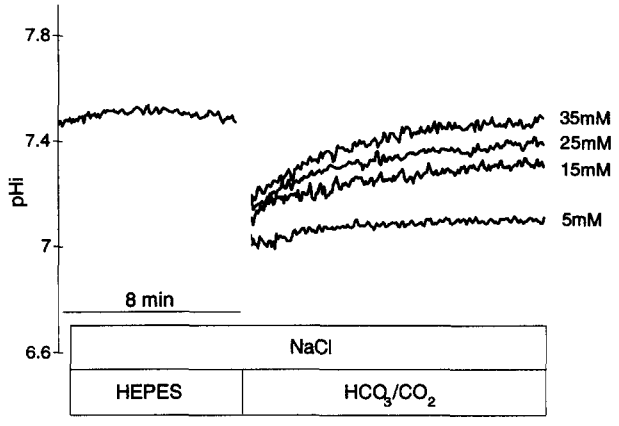

Fig. 3. pH_i recovery in 5% CO_2 shows bicarbonate- and pH_o-sensitivity. Rapid intracellular acidification occurs after switching from Hepes- to HCO_3^-/CO_2-buffer. 5 mM external HCO_3^- ($pH_o = 6.73$) prevents pH_i recovery, while 15 mM HCO_3^- ($pH_o = 7.21$) partially stimulates and 35 mM HCO_3^- ($pH_o = 7.58$) overstimulates pH_i recovery as compared to control conditions (25 mM HCO_3^-; $pH_o = 7.43$). The overlay graph shows traces each of which is representative of six similar experiments.

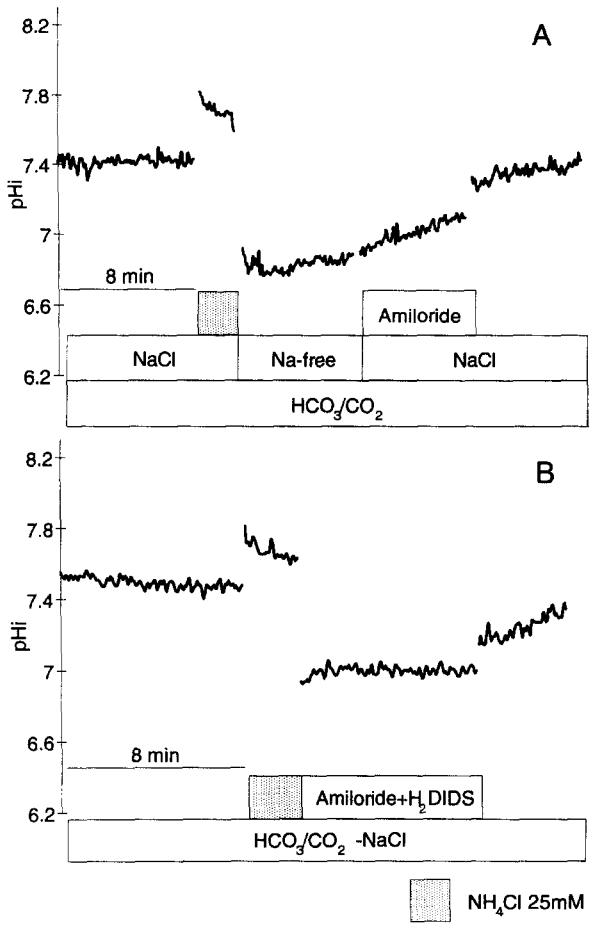

Fig. 4. Influence of Na^+-free solution, amiloride (1 mM) and H_2DIDS (200 μM) on acid-extrusion in HCO_3^-/CO_2-buffer. Cells were acidified by ammonium-prepulse and no recovery occurred under Na^+-free conditions (A). When Na^+ was added back under the presence of amiloride, pH_i recovery occurred, but was partially inhibited. Removal of amiloride led to complete recovery (A). Simultaneous incubation of acidified cells with amiloride and H_2DIDS completely blocked pH_i recovery (B). Traces are representative of six similar experiments.

gated in Hepes-buffer. Na^+-free treatment caused pH_i to decline by 0.19 ± 0.04 pH units over 16 min. Amiloride caused pH_i to decrease by < 0.02 pH units over 1 min, and 0.10 ± 0.02 pH units during 8 min. These data are consistent with the existence of a Na^+/H^+ exchanger, that operates at a slow, but finite rate in the steady state to prevent cellular acidification.

3.3. Evidence for Na^+-HCO_3^- cotransport

Intracellular acid loading was induced either by changing from Hepes- to HCO_3^-/CO_2-buffered medium (Fig. 3) or by performing the ammonium pulse technique in HCO_3^--NaCl (Fig. 4). In both conditions the cells rapidly recovered pH_i in the presence of Na^+ and HCO_3^-. As shown in Fig. 3, the rate of pH_i recovery was dependent on the $[HCO_3^-]$ (and/or pH) of the bathing solutions. The HCO_3^- dependence of pH_i recovery will be discussed in more detail below. As shown in Fig. 4, pH_i recovery did not occur in the absence of Na^+: pH_i stayed at 6.80 ± 0.02 following the pulse with 25 mM NH_4^+. Similarly, switching from Hepes-NaCl to HCO_3^--NMGCl caused pH_i to decrease from ≈ 7.53 to 6.90 ± 0.03, and cells remained acidic (pH_i 6.91 ± 0.03) for 8 min.

When cells were acidified and then switched to HCO_3^--NaCl solution containing 1 mM amiloride, pH_i recovered slowly from 6.83 ± 0.01 to 7.0 ± 0.04 (Fig. 4A). Removal of amiloride caused pH_i to increase more rapidly back to 7.33 ± 0.05. In contrast, when the acidified cells were treated with both 1 mM amiloride and 200 μM H_2DIDS, a specific non-fluorescent inhibitor of HCO_3^--transporting mechanisms [14], pH_i recovery was completely blocked at 6.87 ± 0.02 for 8 min (Fig. 4B). Similar results were obtained when cells were acidified by the introduction of HCO_3^-/CO_2 (data not shown).

In order to evaluate a possible effect of Cl^- on this recovery we performed the same type of experiment in Cl^--free solution. As shown in Fig. 5A, cells recovered from acid loading in the absence of external Cl^-. H_2DIDS partially blocked (Fig. 5B) and amiloride and H_2DIDS completely blocked pH_i recovery (Fig. 5C), indicating that Cl^--independent mechanism(s) were mainly responsible for recovery from an acid load. These data indicate that pH_i recovery occurred by the combined operation of Na^+/H^+ exchange and Na^+-HCO_3^- cotransport [10]: the activity of the Na^+/H^+ exchanger leads to the pH_i rise in Cl^--free solutions containing H_2DIDS; the Na^+-HCO_3^- cotransporter can elicit pH_i recovery in the presence of amiloride; complete inhibition of both mechanisms was achieved by the combined actions of amiloride and H_2DIDS in Cl^--free bathing solution.

We also tested the effect of inverting the transmembrane Na^+ gradient by changing to Na^+-free HCO_3^-/CO_2-solution, and the cells exhibited a pH_i decline of 0.15 ± 0.01 pH-units over the course of 8 min (not shown).

We evaluated the relative abilities of these two transporters to affect pH_i recovery from acid loads by calculating H^+-efflux rates (J^H) at different pH_i values in Hepes-buffered and HCO_3^-/CO_2-buffered solutions (both had pH_o 7.4). Slopes of pH_i recovery were measured at pH_i 7.1, 7.2 and 7.3, and J^H was calculated as described in Section 2. pH_i recovery rates were assumed to be due solely to Na^+/H^+ exchange in Hepes-buffer. The combined rates of the Na^+/H^+ exchanger and Na^+-HCO_3^- cotransporter were calculated from rates of pH_i recovery in HCO_3^-/CO_2-buffered solutions, and these rates were dependent on pH_i (Fig. 6). Subtraction of the Na^+/H^+ exchange rate from the total yielded rates of pH_i recovery due to the Na^+-HCO_3^- cotransporter. As shown in Fig. 6, the J^H due to Na^+/H^+ exchange was sensitive to pH_i, while that due to Na^+-HCO_3^- cotransport was independent of pH_i.

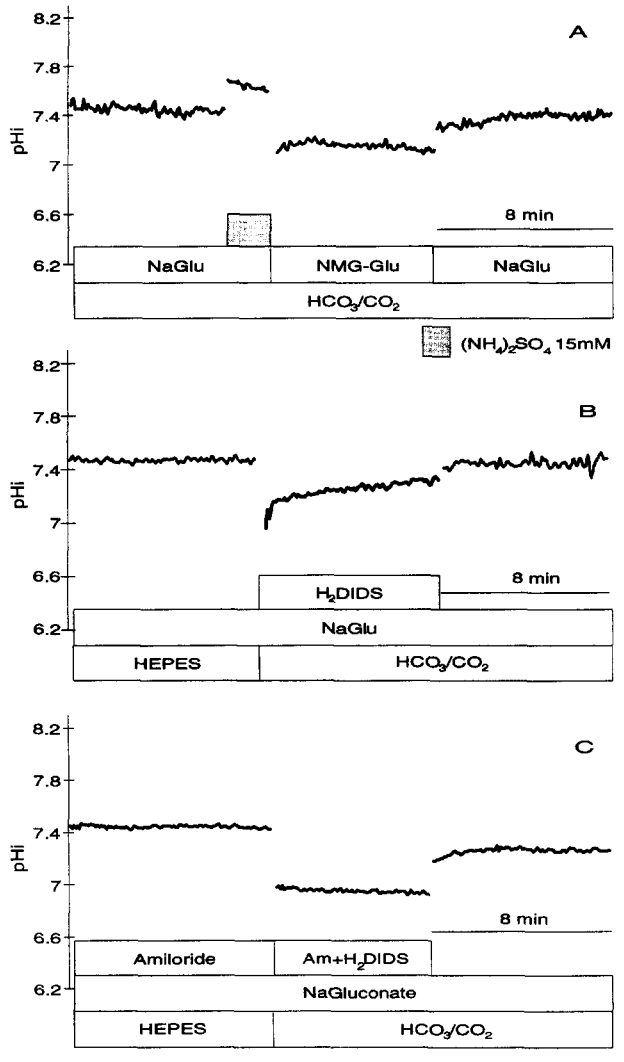

Fig. 5. pH_i recovery from an acid-load in the absence of external Cl^- in HCO_3^-/CO_2 buffer. An ammonium-prepulse was performed with 15 mM $(NH_4)_2SO_4$ in Cl^--free medium. pH_i recovery was completely inhibited in Na^+- and Cl^--free solution; readdition of Na^+ caused total pH_i recovery (A). After intracellular acid-loading by changing from Hepes- to HCO_3^-/CO_2-buffer acid-extrusion occurred in Cl^--free solution containing 200 μM H_2DIDS, but was not complete after 8 min. Removal of H_2DIDS led to complete recovery (B). The combined activities of amiloride and H_2DIDS totally blocked recovery under Cl^--free conditions (C). Traces are representative of five similar experiments.

3.4. Lack of Cl^--dependent pH_i regulation

The possible role of a Cl^-/HCO_3^- exchanger, which in many cells operates by extruding HCO_3^- in exchange for Cl^- in order to dispose of cytoplasmic alkaline loads, can be demonstrated by removal of Cl^- from the bathing solution [24]. Intracellular Cl^- must now leave the cell and HCO_3^- should enter through the anion exchanger, leading to cytoplasmic alkalinization and an increase of pH_i.

When SW-620 cells were incubated in Cl^--free HCO_3^--media (either Na^+-gluconate or Na^+-cyclamate), no pH_i increase was observed. pH_i was 7.47 ± 0.02 in

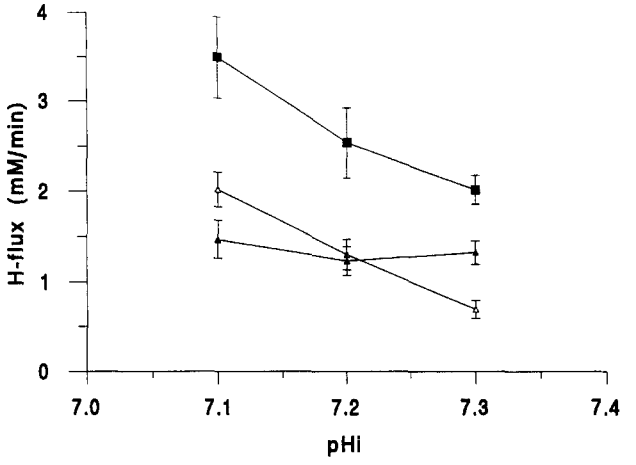

Fig. 6. Relative rates of H^+-extruding mechanisms. Net H^+-flux rates at different pH_i-values are shown for recovery in Hepes-NaCl after ammonium prepulse, reflecting Na^+/H^+ exchange (open triangles; $n = 4$), and in HCO_3^-/CO_2-NaCl after ammonium prepulse (filled squares; $n = 5$), reflecting the combined ativities of Na^+/H^+ exchange and Na^+-HCO_3^- cotransport (filled triangles).

HCO_3^--NaCl and 7.50 ± 0.02 in Cl^--free solution (P = n.s.) (Fig. 7). There were no differences between standard incubation times of 8 min and prolonged exposure of cells to Cl^--free medium over 30 min in order to ensure intracellular Cl^--depletion (data not shown).

This lack of effect of Cl^--free medium on pH_i was not due to the cells having very low intracellular [Cl^-], because addition of 5 μM tributyltin bromide, an artificial anion/OH^- exchanger [1], to Cl^--free HCO_3^--buffer caused pH_i to increase rapidly from 7.43 ± 0.05 to 7.63 ± 0.09 (Fig. 8). Changing back to standard Cl^--containing HCO_3^--buffer produced a steep decline of pH_i and a new resting value of 7.17 ± 0.08.

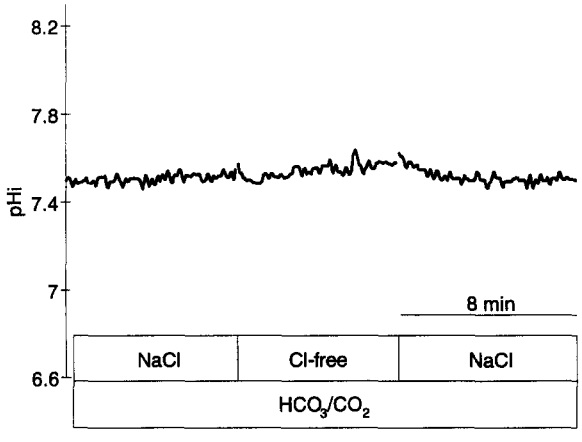

Fig. 7. Exposure to Cl^--free medium in HCO_3^-/CO_2-buffer. When NaCl was isoosmotically replaced by Na^+-cyclamate (or -gluconate, trace not shown), pH_i stayed at basal values. No alkalinization occurred as would be expected in the presence of an operating Cl^-/HCO_3^- exchanger. Trace is representative of 10 similar experiments.

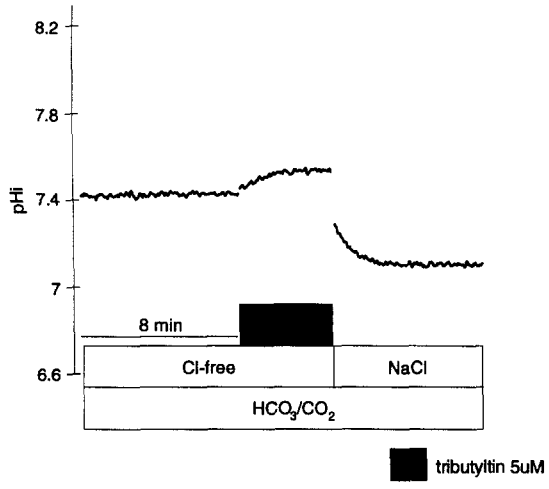

Fig. 8. Effect of insertion of anion exchange with tributyltin. After addition of 5 μM tributyltin bromide to Cl^--free HCO_3^-/CO_2-buffer intracellular alkalinization occurred. Readdition of Cl^- caused pH_i to decrease to a lower resting value. Trace is representative of five similar experiments.

3.5. Influence of pH_o in different HCO_3^-/CO_2 on steady-state pH_i, pH_i recovery and cellular growth

Extracellular pH in tumors may be quite variable depending on metabolic activity and blood flow and we were interested in investigating the effects of different pH_o on SW-620 cells to approximate fluctuating tumor conditions.

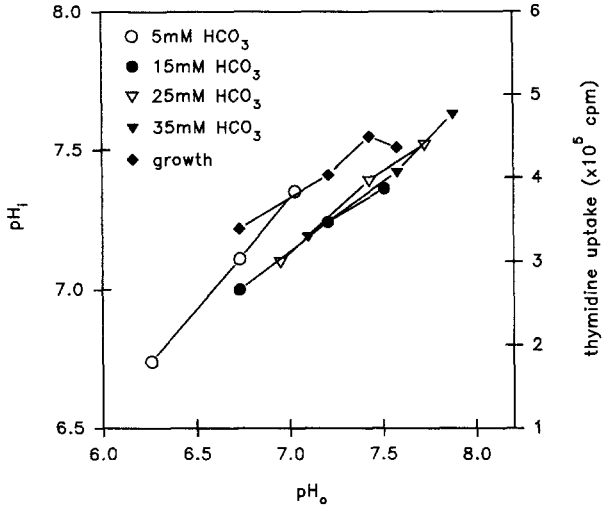

Fig. 9. Summary comparison of the effects of pH_o on steady-state pH_i and growth. pH_o ranging from 6.26 to 7.88 was created by increasing buffer $[HCO_3^-]$ from 5 mM to 35 mM and $[CO_2]$ from 2.5% to 15%. According pH_i values, obtained after a 15 min recovery period from intracellular acidification (same protocol as in Fig. 3), are shown for each $[HCO_3^-]$ at 2.5%, 5% and 15% CO_2 (left, middle and right symbol, respectively). Proliferative activity at different HCO_3^--concentrations and 5% CO_2 was assessed by [^{3}H]thymidine uptake (filled diamonds) and showed significant growth increase with increasing HCO_3^- up to 25 mM, and a slight decrease at higher HCO_3^- (Test for Quadratic Effects). Values represent means of six similar experiments (all S.E.M. < 0.05, not shown).

As shown in Fig. 3, rates and extent of pH_i recovery following acidification with 5% CO_2 depended on $[HCO_3^-]_o$, though, since changes of $[HCO_3^-]_o$ necessarily cause changes of pH_o, it was impossible to determine whether the effects were due to pH_o and/or $[HCO_3^-]_o$. We investigated this phenomenon further by incubating cells in solutions with different $[HCO_3^-]$ (from 5 to 35 mM), CO_2 (from 2.5% to 15%) and pH_o (from 6.26 to 7.88). The effects on steady-state pH_i and growth are summarized in Fig. 9. As shown, pH_i appeared to depend critically on pH_o, and was relatively independent of $[HCO_3^-]$ or $[CO_2]$. In 5% CO_2, cell growth was roughly linearly related to pH_o and pH_i. The simplest interpretation is that steady-state pH_i and growth were both critically dependent on pH_o, indicating that conditions within a tumor may markedly affect cell growth.

4. Discussion

Our data indicate that in SW-620 colon carcinoma cells resting intracellular pH is higher than in other known gastrointestinal cell types, e.g., parietal cells ($pH_i \approx 7.1$, Refs. [20,36]), ileal IEC-6 cells ($pH_i = 7.25$ in Hepes and 7.12 in HCO_3^-/CO_2-buffer, Ref. [37]), and normal colonic epithelial cells isolated from rats ($pH_i \approx 7.0$, Ref. [10]), rabbits ($pH_i \approx 7.23$, Ref. [4]) or humans ($pH_i \approx 7.35$, Ref. [29]). Differences of incubation temperatures probably do not explain the full extent of variability. Following intracellular acidification in Hepes buffer, pH_i recovery was dependent on external Na^+, blocked by amiloride and therefore most likely mediated by a Na^+/H^+ exchanger. The mechanism is reversible, demonstrated by the pH_i decrease in Na^+-free medium. It has been well described and found to be present in most investigated mammalian cells, both in normal and in malignant cell types [11]. The Na^+/H^+ exchanger in SW-620 cells, like that in other cell types, exhibited increased activity as pH_i decreased. Because amiloride caused only a small and very slow decrease of pH_i when added to control cells in Hepes buffer, it appeared likely that the exchanger was operating at a very low rate in the steady state in control cells with $pH_i = 7.53$.

When changing from Hepes- to HCO_3^-/CO_2-buffered medium, acute acid-loading of the human colon carcinoma cells occurred, probably due to the quick entry of CO_2 into the cytoplasm. Subsequently the cells rapidly recovered to a slightly lower pH_i than before in Hepes-buffer. This pH_i recovery was dependent on external Na^+, unaffected by removal of external Cl^- and completely blocked by the combined activities of amiloride and H_2DIDS. The differences in net H^+-efflux during pH_i recovery in Hepes- vs. HCO_3^-/CO_2-containing media indicated the presence of an additional acid extruder in HCO_3^-/CO_2-buffer. Because of its dependence on HCO_3^- and Na^+, the independence from

Cl^- and the inhibition by H_2DIDS, this additional acid extruding mechanism besides the existing Na^+/H^+ exchanger was most likely a Na^+-HCO_3^- cotransporter. These findings are consistent with investigations on IEC-6 cells, a crypt-like rat intestinal cell line [37], rabbit parietal cells [20,32] and rat hepatoma cells [35]. In each of these cell types a *base-loading* Na^+-HCO_3^- cotransporter was described, whereas an *acid-loading* Na^+-HCO_3^- symporter was found in rat glomerular mesangial cells [7,8] and in rabbit renal cortex vesicles [27]. Although it is difficult to determine definitively, it appears likely that the Na^+-HCO_3^- cotransporter works as a base-loading mechanism in SW-620 cells. Thus, the Na^+/H^+ exchanger exhibited only low rates of pH_i regulatory activity at pH_i 7.3–7.5 (Fig. 6; also see above), yet cells were capable of pH_i regulation over this range in HCO_3^-/CO_2-containing media, even in the presence of amiloride (Figs. 4 and 6).

In many cells the acid-extruding Na^+/H^+ exchanger and Na^+-HCO_3^- cotransporter are balanced by an acid-loading anion exchanger, transporting intracellular HCO_3^- (or OH^-) ions for extracellular Cl^-. This Cl^-/HCO_3^- exchanger is located at the basolateral membranes of oxyntic cells in intact sheets of frog gastric mucosa [38] and of HT-29 colonic carcinoma cells grown on filters [9].

In our experiments with SW-620 cells replacement of NaCl with Na^+-gluconate or Na^+-cyclamate in bicarbonate-buffer did not cause cytoplasmic alkalinization, arguing against the presence of an operating anion exchanger. After insertion of anion/OH^- exchange by use of the ionophore tributyltin we observed a marked decrease in pH_i of > 0.2 pH-units. Other authors have similarly found no evidence for anion-exchange in human epidermoid carcinoma A-431cells [22] and rabbit renal OMCD cells [12]. Interestingly, freshly isolated human colonic crypt cells express Na^+-dependent anion exchange [29].

It has been shown in erythrocytes as well as in several epithelia that band 3-immunoreactive or related proteins are involved in Cl^-/HCO_3^- exchange [30]. It seems likely therefore, that the anion exchanger-proteins all belong to a family of proteins related to the erythrocyte band 3-gene. In the SW-620 colon carcinoma cells an altered or repressed expression of the gene responsible for Cl^-/HCO_3^- exchange could account for our observations under Cl^--free conditions. The absence of the anion exchanger in the presence of the base-loading Na^+/H^+ exchanger and Na^+-HCO_3^- cotransporter might then explain why SW-620 colonic carcinoma cells have a higher steady-state pH_i than cells from normal crypts (which exhibit Cl^-/HCO_3^- exchange). It remains unclear, though, whether the alkaline shift of steady-state pH_i is necessary for the transformed function of the tumor cells, for example in terms of a counterregulation against acidic interstitial pH in hypoxic tumor regions, or whether it is just the result of the altered balance between acid- (or alkali-) loading and -extruding mechanisms. Although the genesis of the elevated pH_i in SW-620 cells remains uncertain, it is clear from Fig. 9 that cells grew faster as pH_o and pH_i increased. These data have interesting implications: first, the effects of altered HCO_3^- and CO_2 on steady-state pH_i were both exerted through changes of pH_o (and not through effects of their own), indicating that pH_o plays a critical role in setting pH_i. Second, in tumors with different blood supplies and metabolic activities and resulting pH_o, there will be feedback effects on pH_i and cell growth of the cells in the tumor.

In summary, steady-state pH_i in SW-620 cells in culture is unusually high (7.4–7.5), possibly due to the fact that the cells have two base-loading mechanisms (Na^+/H^+ exchange and Na^+-HCO_3^- cotransport), but, unlike cultured normal intestinal crypt-like cells (IEC-6) and freshly isolated normal colonic crypt cells, SW-620 cells do not have an operating acid-loading anion exchanger at the given conditions. Rates of SW-620 cell growth in culture appear to be controlled by steady-state pH_i, and pH_i seems to be regulated both by the activities of the two base-loading mechanisms and by pH_o. In the in vivo state, rates of growth of individual cells will therefore depend on both the cells' own inherent metabolic and pH_i regulatory activity and, perhaps even more importantly, also on pH_o, which will be determined by the ensemble activity of all the cells of the tumor, including those regulating the blood supply. Our data therefore indicate that manipulation of pH_i regulatory mechanisms (either blocking the base loaders or obtaining expression of an acid loader) in combination with maneuvers designed to alter pH_o might have clinical relevance for treatment of colorectal cancer.

Acknowledgements

Support by 'Wissenschaftlicher Fonds des Bürgermeisters der Stadt Wien' and 'Jubiläumsfonds der Österreichischen Nationalbank'. The authors thank Maria Prettenhofer and Anna Zomer for excellent technical assistance and Dr. Martina Mittlböck for performing statistical analysis.

References

[1] Antonenko, Y. (1990) Electrically silent anion transport through bilayer lipid membrane induced by tributyltin and triethyllead. J. Membr. Biol. 113, 109–113.
[2] Benedetti, A., Baroni, G.S., Marucci, L., Mancini, R., Jezequel, A.M. and Orlandi, F. (1993) Regulation of intracellular pH in isolated periportal and perivenular rat hepatocytes. Gastroenterology 105, 1797–1805.
[3] Benos, D.J. (1982) Amiloride: a molecular probe of sodium transport in tissues and cells. Am. J. Physiol. 242, C131–C145.
[4] Benya, R.V., Schmidt, L.N., Sahi, J., Layden, T.J., Rao, M.C. (1991) Isolation, characterization, and attachment of rabbit distal colon epithelial cells. Gastroenterology 101, 692–702.
[5] Bierman, A.J., Tertoolen, L.G.J., DeLaat, S.W. and Moolenaar, W.H. (1987) The Na^+/H^+ exchanger is constitutively activated in

P19 embryonal carcinoma cells, but not in a differentiated derivative. J. Biol. Chem. 262, 9621–9628.
[6] Boron, W.F. and Boulpaep, E.L. (1983) Intracellular pH regulation in the renal proximal tubule of the salamander: basolateral HCO_3^- transport. J. Gen. Physiol. 81, 53–94.
[7] Boyarsky, G., Ganz, M.B., Sterzel, R.B. and Boron, W.F. (1988) Intracellular pH regulation in single glomerular mesangial cells. I. Acid extrusion in absence and presence of HCO_3^-. Am. J. Physiol. 255, C844–C856.
[8] Boyarsky, G., Ganz, M.B., Sterzel, R.B. and Boron, W.F. (1988) Intracellular pH regulation in single glomerular mesangial cells. II. Na^+-dependent and -independent Cl^-/HCO_3^- exchanger. Am. J. Physiol. 255, C857–C869.
[9] Busche, R., Jeromin, A., Von Engelhardt, W. and Rechkemmer, G. (1993) Basolateral mechanisms of intracellular pH regulation in the colonic epithelial cell line HT29 clone 19A. Pflügers Arch. 425, 219–224.
[10] Diener, M.C., Helmle-Kolb, C., Murer, H. and Scharrer, E. (1993) Effect of short-chain fatty acids on cell volume and intracellular pH in rat distal colon. Pflügers Arch. 424, 216–223.
[11] Grinstein, S. and Rothstein, A. (1986) Mechanisms of regulation of the Na^+/H^+ exchanger. J. Membr. Biol. 90, 1–12.
[12] Hayashi, M., Schuster, V.L. and Stokes, J.B. (1988) Absence of transepithelial anion exchange by rabbit OMCD: evidence against reversal of cell polarity. Am. J. Physiol. 255, F220–F228.
[13] Leibovitz, A., Stinson, J.C., McCombs, W.B. III, McCoy, C.E., Mazur, K.C. and Mabry, N.D. (1976) Classification of human colorectal adenocarcinoma cell lines. Cancer Res. 36, 4562–4569.
[14] Lepke, S., Fasold, H., Pring, M. and Passow, H.A. (1976) Study of the relationship between the anion exchange and binding to the red cell membrane of 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) and its dihydroderivate H_2DIDS. J. Membr. Biol. 29, 147–177.
[15] Moolenaar, W.H., Tertoolen, L.G.J. and DeLaat, S.W. (1984) Phorbol esters and diacylglycerol mimic growth factors in raising cytoplasmic pH. Nature 312, 371–374.
[16] Moolenaar, W.H., Tsien, R.Y., Van der Saag, P.T. and DeLaat, S.W. (1983) Na^+/H^+ exchange and cytoplasmic pH in the action of growth factors in human fibroblasts. Nature 304, 645–648.
[17] Newell, K.J. and Tannock, I.F. (1989) Reduction of intracellular pH as a possible mechanism for killing cells in acidic regions of solid tumors: effects of carbonyl cyanide 3-chlorophenylhydrazone. Cancer Res. 49, 4477–4482.
[18] Nuccitelli, R. and Helple, J.M. (1982) Intracellular pH: its measurement, regulation, and utilisation in cellular functions, pp. 567–586, Alan R. Liss, New York.
[19] Paradiso, A.M., Negulescu, P.A. and Machen, T.E. (1986) Na^+-H^+ and Cl^--OH^- (HCO_3^-) exchange in gastric glands. Am. J. Physiol. 250, G524–G534.
[20] Paradiso, A.M., Townsley, M.C., Wenzl, E. and Machen, T.E. (1989) Regulation of intracellular pH in resting and stimulated parietal cells. Am. J. Physiol. 257, C554–C561.
[21] Putnam, R.W. and Grubbs, R.D. (1990) Steady-state pH_i, buffering power, and effect of CO_2 in a smooth muscle-like cell line. Am. J. Physiol. 258, C461–C469.
[22] Reinertsen, K.V., Tonessen, T.I., Jacobsen, J., Sandvig, K. and Olsnes, R. (1988) Role of chloride/bicarbonate antiport in the control of cytosolic pH. Cell-line differences in activity and regulation of antiport. J. Biol. Chem. 263, 11117–11125.
[23] Rink, T.J., Tsien, R.Y. and Pozzan, T. (1982) Cytoplasmic pH and free Mg^{2+} in lymphocytes. J. Cell Biol. 95, 189–196.
[24] Roos, A. and Boron, W.F. (1981) Intracellular pH. Physiol. Rev. 61, 296–434.
[25] Rotin, D., Steele-Norwood, D., Grinstein, S., Tannock, I. (1989) Requirement of the Na^+/H^+ exchanger for tumor growth. Cancer Res. 49, 205–211.
[26] Rotin, D., Wan, P., Grinstein, S. and Tannock, I. (1987) Cytotoxicty of compounds that interfere with the regulation of intracellular pH: a potential new class of anticancer drugs. Cancer Res. 47, 1497–1504.
[27] Soleimani, M., Grassl, S.M. and Aronson, P.S. (1987) Stoichiometry of Na^+-HCO_3^- cotransport in basolateral membrane vesicles isolated from rabbit renal cortex. J. Clin. Invest. 79, 1276–1280.
[28] Starlinger, M., Paradiso, A.M. and Machen, T.E. (1987) Steady-state regulation of intracellular pH in isolated rabbit gastric glands. Gastroenterology 92, 957–965.
[29] Teleky, B., Hamilton, G., Cosentini, E., Bischof, G., Riegler, M., Koperna, T., Feil, W., Schiessel, R. and Wenzl, E. (1994) Intracellular pH regulation of human colonic crypt cells. Pflügers Arch. 426, 267–275.
[30] Thomas, H.A., Machen, T.E., Smolka, A., Baron, R. and Kopito, R. (1989) Identification of a 185-kDa band 3-related polypeptide in oxynctic cells. Am. J. Physiol. 257, C537–C544.
[31] Thomas, J.A., Buchsbaum, R.N., Zimniak, A. and Racker, E. (1979) Intracellular pH measurement in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. Biochemistry 18, 2210–2218.
[32] Townsley, M.C. and Machen, T.E. (1989) Na^+-HCO_3^- cotransport in rabbit parietal cells. Am. J. Physiol. 257, G350–G356.
[33] Trainer, D.L., Kline, T., McCabe, F.L., Faucette, L.F., Feild, J., Chaikin, M., Anzano, M., Rieman, D., Hoffstein, S., Li, D.J., Gennaro, D., Buscarino, C., Lynch, M., Poste, G. and Greig, R. (1988) Biological characterization and oncogene expression in human colorectal carcinoma cell lines. Int. J. Cancer 41, 287–296.
[34] Wagner, J.D., Kurtin, P. and Charney, A.N. (1985) Effect of systemic acid-base disorders on colonic intracellular pH and ion transport. Am. J. Physiol. 249, G39–G47.
[35] Weintraub, W.H. and Machen, T.E. (1989) pH regulation in hepatoma cells: roles for Na^+-H^+ exchange, Cl^--HCO_3^- exchange, and Na^+-HCO_3^- cotransport. Am. J. Physiol. 257, G317–G327.
[36] Wenzl, E. and Machen, T.E. (1989) pH_i dependence of buffer capacity and anion exchange in the parietal cell. Am. J. Physiol. 257, G741–G747.
[37] Wenzl, E., Sjaastadt, M.D., Weintraub, W.H. and Machen, T.E. (1989) Intracellular pH regulation in IEC-6 cells, a cryptlike intestinal cell line. Am. J. Physiol. 257, G732–G740.
[38] Yanaka, A., Carter, K.J., Lee, H.H. and Silen, W. (1990) Influence of Cl^- on pH_i in oxynticopeptic cells of in vitro frog gastric mucosa. Am. J. Physiol. 258, G815–G824.