Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 7414–7418, October 1990
Cell Biology

Tumorigenic 3T3 cells maintain an alkaline intracellular pH under physiological conditions

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Communicated by Efraim Racker, June 18, 1990 (received for review April 4, 1990)

ABSTRACT **One of the earliest events in the response of mammalian cells to mitogens is activation of Na^+/H^+ exchange, which increases intracellular pH (pH^{in}) in the absence of HCO_3^- or at external pH values below 7.2. The proliferative response can be blocked by preventing the pH^{in} increase; yet, the proliferative response cannot be stimulated by artificially raising pH^{in} with weak bases or high medium pH. These observations support the hypothesis that optimal pH^{in} is a necessary, but not sufficient, component of the proliferative-response sequence. This hypothesis has recently been challenged by the observation that transfection of NIH 3T3 cells with yeast H^+-ATPase renders them tumorigenic. Although previous measurements indicated that these transfected cells maintain a higher pH^{in} in the absence of HCO_3^-, whether H^+-ATPase transfection raised the pH^{in} under physiologically relevant conditions was not known. The current report shows that these transfected cells do maintain a higher pH^{in} than control cells in the presence of HCO_3^-, supporting the possibility that elevated pH^{in} is a proliferative trigger *in situ*. We also show that these cells are serum-independent for growth and that they glycolyze much more rapidly than phenotypically normal cells.**

All mammalian cells maintain a higher intracellular pH (pH^{in}) than predicted from Nernst equilibrium (1). Maintenance of this pH gradient can be mediated by three transport systems: Na^+/H^+ exchange, HCO_3^- transport, and plasma membrane H^+-ATPase. Most, if not all, cells contain Na^+/H^+ exchange and HCO_3^- transport activities, whereas plasma membrane H^+-ATPase activity is generally observed only in specialized epithelia, such as bladder, intestine, and kidney (2). Interplay between these two (and sometimes three) systems buffers the pH^{in} against changes in the extracellular pH (pH^{ex}) and can mediate changes in pH^{in} during certain physiological responses.

One of the earliest events in the response of mammalian cells to mitogens is activation of Na^+/H^+ exchange (3, 4). In the absence of HCO_3^- or at low pH^{ex}, this increases pH^{in} (5, 6). Although it was earlier thought that elevated pH^{in} might be a second messenger in the proliferative response (7, 8), current hypotheses regard elevated pH^{in} as only permissive for proliferation (4, 5). To assign a causal role for pH^{in} in proliferation, elevated pH^{in} must be seen upon stimulation, inhibition of the increase in pH^{in} must inhibit proliferation, and mimicking the increase must be sufficient to stimulate proliferation. These conditions are not satisfied *in vitro*. Although preventing the increase in pH^{in} generally inhibits proliferation (3, 4, 9), many cells do not increase their pH^{in} at high pH^{ex} (e.g., pH 7.4) in the presence of bicarbonate (5, 6, 10, 11), and artificially raising pH^{in} with weak bases or high pH^{ex} is not sufficient to stimulate proliferation (12, 13). It is possible, however, that these conditions are satisfied *in vivo*. For instance, we and others have shown that mammalian cells will alkalinize upon mitogenic stimulation under conditions expected *in vivo* (e.g., pH 7.0–7.2) (cf. refs. 5, 6, and 14).

Additionally, Perona and Serrano have demonstrated that *in vivo* tumorigenicity can be induced in phenotypically normal 3T3 mouse embryo cells by transfection with yeast H^+-ATPase (15). These cells clone in soft agar, grow to higher density, and are tumorigenic in nude mice. There are a number of consequences of this transfection which may be important to the tumorigenic response—such as elevated pH^{in}, increased membrane potential (16), or increased rate of ATP turnover. Although the activity of the H^+-ATPase could be expected to increase pH^{in}, there are two other H^+-transporting systems in these cells (e.g., Na^+/H^+ exchange; Na^+-dependent Cl^-/HCO_3^- exchange) the activity of which could override effects of the H^+-ATPase. A fundamentally important and unresolved question in this system is whether the pH^{in} of these tumorigenic cells is higher under physiologically relevant conditions.

In the present communication, we accurately measure the pH^{in} of these cells vis-a-vis normal 3T3 cells and cells transfected with an inactive form of the H^+-ATPase and report that the pH^{in} of the tumorigenic cells is significantly more alkaline than that of the normal cells under physiological conditions. We have also examined the effects that this altered pH^{in} may have on cellular physiology that is germane to the transformed phenotype. These tumorigenic cells do not grow faster than their normal counterparts under normal culture conditions. However, they do grow to much higher densities and have lost their serum requirement for growth. We have also observed that these cells exhibit a rapid rate of aerobic glycolysis and that this increased activity cannot be accounted for by the elevated pH^{in} alone. The cause of tumorigenicity in these cells could lie in their altered regulation of pH^{in}, which may manifest itself through serum-independence, and elevated pH^{in} may be a proliferative trigger *in vivo*.

MATERIALS AND METHODS

Cells. RN1a cells were produced as described (15, 16). Briefly, 500,000 NIH 3T3 cells were transfected using 0.1 μg of pSV_2neo plasmid, 8 μg of carrier DNA, and 10 μg of pSV_hAT_5 plasmid, which contained the gene for the yeast plasma membrane H^+-ATPase (allele *PMA1*) under control of the simian virus 40 promoter. Clones were selected with G-418

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Abbreviations: pH^{in}, intracellular pH; pH^{ex}, extracellular pH; SNARF-1, *N*,*N*-dimethyl-1,5,6-biscarboxyseminaphthorhodafluor; BCECF, 2′,7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein; CSB, cell suspension buffer; HBSS, Hanks' balanced salt solution.

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and isolated by cylinder cloning. N-mut cells were prepared in the same way but contain allele *pma213*: a $Glu_{233} \rightarrow Gln$ mutation that gives rise to an "uncoupled" form of the H^+-ATPase, with 40% of the ATPase activity and 20% of the H^+-pumping activity as compared with *PMA1* (16, 17). BALB/c 3T3 mouse embryo fibroblasts were obtained from American Type Culture Collection (ATCC CCL 163). NIH 3T3 cells were gifts from Greg Duester (Colorado State University, Fort Collins, CO). Although NIH 3T3 cells were the parent line for all transfectants, we also used BALB/c 3T3 cells as controls in this study because they are much better characterized. Previous studies in our lab have shown no differences in the pH^{in} regulation between NIH and BALB/c cells (18).

All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO), supplemented with 10% Nu-Serum (Collaborative Research). The initial inocula as received were grown to 70% confluency in 300 cm^2, at which time the cells were frozen in DMEM/10% dimethyl sulfoxide/20% Nu-Serum at a density of 1×10^6 cells per freezing ampule. Cells from these frozen stocks were recovered every 6 weeks in 75-cm^2 tissue culture flasks and passed bi-weekly at an inoculation density of 2×10^5 cells per 75-cm^2 flask. Cells were subcultured for 2 weeks before use in experiments.

For fluorescence experiments, cells were plated into Petri dishes containing 9×22 mm coverslips at a density of 10^6 cells per dish in DMEM/10% Nu-Serum. On the following day, these cultures were prepared for fluorescence determination of pH^{in} as described below. For other experiments, cells were consistently plated at a density of 5×10^3 cells per cm^2.

Chemicals and Buffers. All dyes were purchased from Molecular Probes. Amiloride, nigericin, and valinomycin were all purchased from Sigma.

Cell Suspension Buffer (CSB). CSB contained 1.3 mM $CaCl_2$/1 mM $MgSO_4$/5.4 mM KCl/0.44 mM KH_2PO_4/110 mM NaCl/0.35 mM Na_2HPO_4/24 mM $NaHCO_3$/5 mM glucose/2 mM glutamine. The pH of CSB and media was maintained with 50 mM Mes/Hepes/Tricine. For HCO_3^--free CSB, 24 mM NaCl was substituted for the $NaHCO_3^-$ in CSB. Hanks' balanced salt solution (HBSS) was 138 mM NaCl/1 mM $CaCl_2$/1 mM $MgCl_2$/5.4 mM KCl/0.35 mM Na_2HPO_4/5 mM glucose/2 mM glutamine/0.03 mM phenol red. The salt components of all buffers were prepared at least a day before experiments to allow for equilibrium hydration of CO_2, whereas the organic components were always added immediately before experiments to avoid bacterial contamination.

Measurement of pH^{in}. Intracellular pH was determined by fluorescence of either *N*,*N*-dimethyl-1,5,6-biscarboxyseminaphthorhodofluor SNARF-1 or 2',7'-bis(2-carboxyethyl)-5,6-carboxyfluorescein (BCECF). The dyes were loaded into cells grown on coverslips in their acetoxymethyl ester forms, as described (19). Briefly, coverslips were incubated for 30 min at 37°C in CSB containing 20 μM SNARF-1 or 2 μM BCECF in their respective acetoxymethyl ester forms. After this, cells were rinsed three times with CSB and incubated 30 more min to ensure complete ester hydrolysis. Fluorescence was determined with an SLM-8000C spectrofluorometer outfitted with a flowthrough device for sample perfusion (20). Two coverslips containing cells were mounted in the holder back to back and were continuously perfused at 1 ml/min. Sample temperature was maintained at 37°C by keeping both the water jacket and the perfusion medium at 37°C. The fluorescence-ratio methods used for these dyes are described elsewhere (18, 19, 21).

Measurement of Acid Production. Cells were plated into 96-well microtiter plates (Flow Laboratories) and allowed to grow to complete confluency (≈2 days). At the time of experiment, medium was aspirated and wells were washed three times with unbuffered HBSS. Cells were then incubated in 100 μl of unbuffered HBSS, and the absorbance of phenol red was monitored at 450 nm and 490 nm for 30 min by using a microtiter plate reader (Bio-Tek, Burlington, VT). The ratio of 450/490 absorbance can be converted to pH by using the equation:

$$pH = pK_a + \log [(R - R_{min})/(R_{max} - R)], \quad [1]$$

where R is the measured ratio of absorbance at two wavelengths, R_{max} is the ratio of the anion form of dye, and R_{min} is the ratio of the protonated dye. In the case of phenol red, $pK_a = 7.50$, $R_{min} = 0.2$, and $R_{max} = 2.5$. The dpH/dt was calculated and converted to H^+ equivalents by correcting for buffering capacities, which were 330 μM H^+ per pH unit for amiloride-containing solutions and 147 μM H^+ per pH unit for amiloride-free solutions, as determined by titration of stock solutions.

Previously Described Analyses. Glucose and lactate concentrations in media were monitored with an automated, simultaneous glucose/lactate analyzer (Yellow Springs Instruments). H^+-ATPase protein was measured by immunofluorescence, and the presence of H^+-ATPase DNA was monitored by Southern analysis (15). Cell mass was determined by crystal violet staining and converted to cell number, as described (22). Intracellular K^+ was determined by atomic absorption spectroscopy (23). Least-squares analysis and simplex curve fitting were done with MINSQ (MicroMath Software).

RESULTS

Dye Calibration. Measurement of absolute pH^{in} for comparison between different cell types is not simple, although the process is straightforward. The most sensitive method for measuring pH^{in} in mammalian cells uses fluorescence ratios (21). However, dye characteristics can be affected by the composition of the internal milieu (24). To correct for potential artifacts, the behavior of each dye must be accurately determined *in situ* for each cell type examined. Examples of these data are illustrated in Fig. 1 *A* and *B*, where we express the pH dependence of fluorescence ratios for two dyes: SNARF-1 and BCECF in three types of cells: RN1a cells transfected with wild-type allele, N-mut cells transfected with mutant allele *pma213*, and normal BALB/c 3T3 cells.

The pH gradients of these cells have been collapsed by using a combination of 147 mM KCl, 2 μM valinomycin, and 6.8 μM nigericin. The high K^+ is used to approximate intracellular K^+ concentration, as determined by atomic absorption. Valinomycin completes the collapse of the K^+ gradient without significant effects on cell volume, and nigericin sets the H^+ gradient equal to the K^+ gradient, which, in this case, is unity. As illustrated, the ratios of both SNARF-1 and BCECF are sensitive to pH. Ratios at values >pH 8.0 are not obtainable, as discussed (19). By using least-squares analysis, these data are iteratively solved for pKa, R_{min}, and R_{max} in Eq. 1. As shown in Table 1, these values slightly differ between the different cell types (see below).

Intracellular pH. The effects of external pH (pH^{ex}) on the pH^{in} of these cells are illustrated in Fig. 2. These data were obtained by perfusing cells with CSB containing HCO_3^- at concentrations set by equilibrium with 5% CO_2 atmosphere (see Fig. 2 legend). After each determination, cells were perfused with a buffer containing nigericin, valinomycin, and high K^+ at pH 7.2 to obtain a calibration point. These calibrations were used to correlate the observed ratios with the titration curves in Fig. 1. This procedure corrects for minor differences in dye loading between samples (19). In practice, these correction factors were consistently insignif-

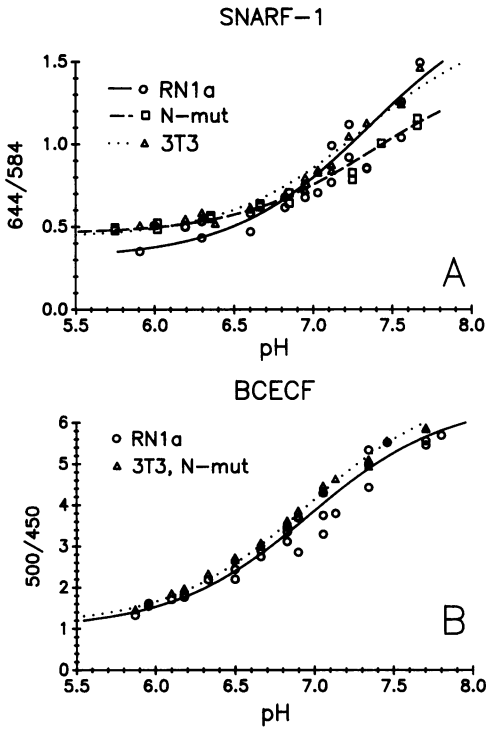

FIG. 1. Fluorescence ratio of intracellularly loaded SNARF-1 and BCECF in 3T3 cells, the pH gradient of which has been collapsed. RN1a, N-mut, and BALB/c 3T3 cells were prepared and loaded with dye, as described. Cells were perfused with buffer containing 147 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 2 μM valinomycin, and 6.8 μM nigericin. Data were collected with an SLM 8000c spectrofluorometer and are expressed as the fluorescence ratio produced at excitation wavelengths of 500 nm and 450 nm for BCECF (λ_{em} = 529 nm) and the fluorescence ratio observed at emission wavelengths of 644 nm and 584 nm for SNARF-1 (λ_{ex} = 534 nm). Buffer pH was determined with a Beckman pHi 72 pH meter and a gel-filled combination glass electrode calibrated at 37°C to pH 7.0 and 10.0 with commercially available solutions (VWR). Solid, dashed, and dotted lines represent data fit to Eq. 1 from data in Table 1 for RN1a, N-mut, and 3T3 cells, respectively.

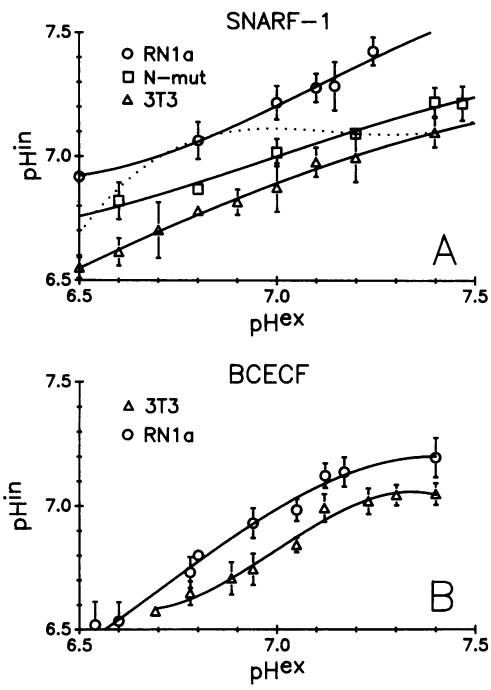

FIG. 2. pH^{in} of RN1a, N-Mut, and BALB/c 3T3 cells as function of pH^{ex}, as calculated from the fluorescence ratios of SNARF-1 (*A*) and BCECF (*B*). Cells were prepared as described in the legend for Fig. 1 and perfused with HBSS without phenol red containing 50 mM organic buffer and HCO_3^-, the concentration of which is set by the buffer pH using the equation: $[HCO_3^-] = (1.52\ mM) \times [10^{(pH-6.24)}]$; where 1.52 mM is the concentration of CO_2 in HBSS at 37°C and 6.24 is the pK_a for the process of CO_2 hydration. These buffers were maintained at 37°C in 5% CO_2 atmosphere for at least 12 hr before experiments. Dotted lines in Fig. *A* represents the pH^{in} of BALB/c 3T3 cells with 10% Nu-Serum (6).

icant. As shown by the data in Fig. 2, the pH^{in} of the transformed RN1a cells, determined with two different dyes, is more alkaline than the pH^{in} of the phenotypically normal cells. By least-squares analysis, the pH^{in} of all three cell types differs significantly to confidence levels of 90% (N-mut vs. 3T3), 95% (N-mut vs. RN1a), and 99% (3T3 vs. RN1a). Notice that there are differences in R_{min} and R_{max} of SNARF-1 fluorescence between the cell types (Table 1). The data presented in Fig. 2 were calculated using individual values for each cell type. If, instead, the median (for SNARF-1) or mean (for BCECF) values are used, the pH^{in} values reported for N-mut and RN1a cells are lower by 0.1–0.15 pH unit. Propagating these values into the data of Fig. 2 eliminates the significance between N-mut and 3T3 cells; yet, both remain significantly different from RN1a cells to a 95% confidence interval. Therefore, by using two dyes and correcting for possible artifacts and propagating errors, we can state with high confidence that the pH^{in} of tumorigenic RN1a cells is significantly higher than that of their nontumorigenic counterparts. A remaining problem is the difference in the absolute pH^{in} determined with the two dyes. Although both dyes consistently show that the pH^{in} of RN1a cells is higher than that of the phenotypically normal cells, SNARF-1 consistently reports a higher pH^{in} relative to that obtained with BCECF. The cause for this discrepancy between the dyes is unknown.

Cell Growth and Serum Requirement. Because the pH^{in} of the RN1a cells is higher than that of 3T3 cells, we might also predict that they would grow at a faster rate because pH^{in}

Table 1. Parameters for estimating pH^{in} by using SNARF-1 and BCECF *in situ*

Cell type	pK_a	R_{min}	R_{max}	*n*
		SNARF-1		
BALB/c	7.394 ± 0.109	**0.442 ± 0.016***	**1.837 ± 0.170**	18
N-Mut	**7.389 ± 0.131**	0.456 ± 0.008*	1.482 ± 0.150†	16
RN1a	7.358 ± 0.156	0.310 ± 0.024‡	1.908 ± 0.307	22
		BCECF		
BALB/c	6.956 ± 0.025	1.081 ± 0.042	6.884 ± 0.077	27
RN1a	6.967 ± 0.061	0.999 ± 0.088‡	6.509 ± 0.195‡	27

Numbers in boldface represent median values. Statistical analysis was done by using Student's *t* test; unreported differences are not significant.
*$P < 0.001$ N-mut versus BALB/c.
†$P < 0.005$ N-mut versus BALB/c or RN1a.
‡$P < 0.0005$ RN1a versus BALB/c cells.

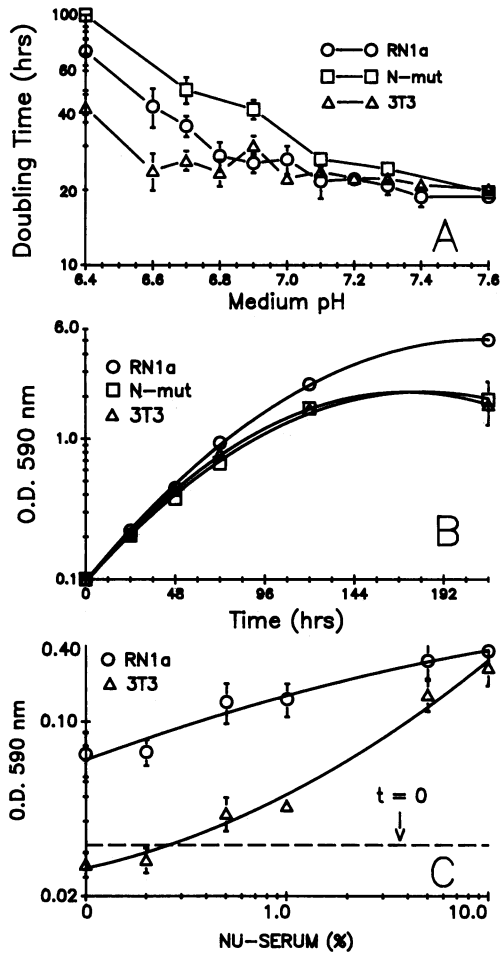

FIG. 3. Growth characteristics of RN1a and 3T3 cells. Data for 3T3 cells are pooled from experiments with BALB/c and NIH 3T3 cells. Cells were grown in 24-well miniwell plates, and cell number was determined by crystal violet staining as has been described. Medium pH was kept constant by using combinations of HCO_3^-, Mes, Hepes, and Tricine; total concentration of nonvolatile buffers was 50 mM. (*A*) Culture doubling time (hr ± SD) as function of medium pH. Cell numbers were determined at various time points, and logarithmic-phase doubling time was determined by fitting data to equation: $N_t = N_0 2^{t/\alpha}$, where N_0 and N_t equal cell numbers at time zero and a given time point, respectively, t equals time in hr, and α equals doubling time. (*B*) Cell density (OD of crystal violet) as function of culture time. If not shown, error bars (SD) are smaller than data points. Line represents second-order polynomial fit. (*C*) Cell growth as function of serum concentration. Cells were plated at density indicated by dashed line and maintained for 72 hr at pH 7.2, after which cell mass was determined by crystal violet staining.

correlates with growth rate in a number of cell types (25); this is not observed. Fig. 3*A* illustrates that, at low density, 3T3 cells grow faster than RN1a cells at pH values below 6.8. Although they grow slower, RN1a cells grow to much higher densities than do 3T3 cells, as evidenced by cell number after 9 days in culture (Fig. 3*B*). The data from higher-density cultures compare favorably with those obtained recently by Perona *et al.* (16).

Significantly, RN1a cells are not dependent upon serum supplementation for growth (Fig. 3*C*). The pH^{in} measurements shown in Fig. 2 were made without serum, and the growth studies of Fig. 3 *A* and *B* were done with 10% Nu-Serum. Nu-Serum addition raises the pH^{in} of 3T3 cells to be similar to that of the RN1a cells at neutral pH (6), illustrated by the dotted line in Fig. 2*A*. Therefore, with serum, we would expect the pH^{in} differences to be less significant.

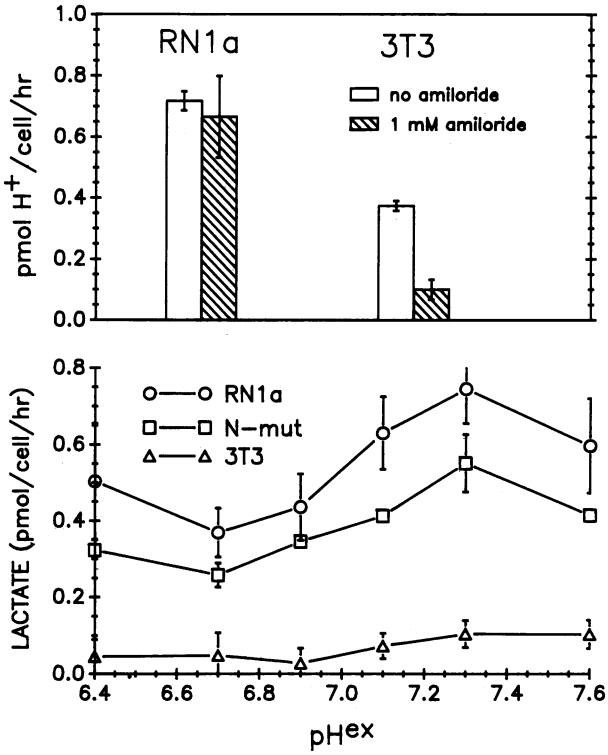

FIG. 4. Acid production by RN1a and 3T3 cells (both BALB/c and NIH). (*Upper*) H^+ production by RN1a and 3T3 cells grown in microtiter plates was determined by monitoring medium pH as function of time, as described. Values are expressed as pmol of H^+ per cell per hr ± SD (n = 8). (*Lower*) Lactate production by RN1a, N-mut, and 3T3 cells grown in 24-well plates was determined by monitoring lactate levels with a lactate analyzer, as described. Cells were all grown at pH = 7.2–7.4 until time of the experiment, when they were washed and incubated with media buffered at indicated pH values with 50 mM Mes/Hepes/Tricine. Samples were analyzed at 30, 60, and 180 min of incubation, and data were fit to a linear equation. Data are expressed as pmol of lactate per cell per hr ± SD (n = 4 for each data point).

Glycolysis and Acid Production. RN1a cells produce much more acid than do 3T3 cells (Fig. 4*A*). Compared with 3T3 cells, the extrusion of H^+ equivalents by RN1a cells is not significantly inhibited by amiloride and lack of HCO_3^-. As shown in Fig. 4*B*, most of this excess acid is lactate. These data indicate that N-mut and RN1a cells glycolyze about 10 times more rapidly than do the 3T3 cells. Although the glycolytic rate of all three cell types increases 2- to 3-fold between pH 6.7 and 7.3, lactate production in 3T3 cells never approaches the high rates seen in N-mut or RN1a cells. This finding suggests that the cause of the high rate of aerobic glycolysis lies in something other than altered pH^{in} regulation.

DISCUSSION

Is altered pH^{in} regulation related to tumorigenesis? Clearly, in this artificial construct, the H^+-ATPase-transfected, tumorigenic RN1a cells have a higher pH^{in} under physiological (e.g., HCO_3^--containing) conditions than do their nontumorigenic counterparts. Although there is no change in the pH^{in} of normal cells in response to mitogens under culture conditions (e.g., pH^{ex} 7.3–7.4), there are significant increases in pH^{in} at the lower pH^{ex} values expected *in vivo* (e.g., 7.0–7.2) (5, 6, 15). These tumorigenic cells, therefore, have a pH^{in} without serum that is higher than that of normal cells with serum. Significantly, these tumor cells are also serum-independent for growth, which raises the possibility that the

supraphysiological pH^{in} circumvents other mitogen-induced events and stimulates growth directly.

Alkalinization might also be involved in natural tumorigenesis. Growth cessation *in vivo* may be induced by absence of growth factors or density-dependent inhibition. These data are consistent with a model wherein a physiological signal for growth cessation is loss of pH^{in} regulation and, hence, intracellular acidification. H^+-ATPases may be refractory to such regulation, allowing otherwise inhibited cells to maintain a high pH^{in} and, therefore, grow.

Could H^+-ATPases be involved in natural carcinogenesis? There is evidence for an electrogenic H^+-exporting ATPase in Ehrlich ascites tumor (EAT) cells (26), enhanced H^+-extruding activity is expressed upon transformation of Chinese hamster ovary cells (27), and ATP is directly required for pH^{in} regulation in both A-431 tumor cells (28) and EAT cells (29). Plasmalemmal H^+-ATPase expressed in the nonepithelial cells might be a plasma membrane type H^+/K^+-ATPase, or it could be a vacuolar-type, as suggested by Racker (30). Pathological expression of a clathrin-coated vesicle or vacuolar H^+-ATPase could arise from something as simple as increased retention time in the plasma membrane during recycling. Vacuolar-type H^+-ATPases have been observed in the plasma membranes of EAT cells (30), osteoclasts (31), and macrophages (32). Overexpression of a plasma membrane H^+-ATPase in nonepithelial cells is, therefore, feasible. The consequences of such expression might lead to tumorigenesis, as evidenced by the current system.

It must be remembered, however, that insertion of a H^+-ATPase into the plasma membrane will induce other effects besides increasing the magnitude of the pH gradient. Because this pump is electrogenic, these cells have a membrane potential of about -40 mV, while the membrane potential of the parent cells is -20 mV (16). Such a difference could cause significant changes in transport thermodynamics, and these changes could contribute to the transformed phenotype. Also, it is likely that these cells have an increased turnover rate of ATP, and this could lead to metabolic changes corresponding to transformation.

An example of this is glycolysis, which is probably a consequence of high ATPase rates. High rates of glycolysis are seen in both RN1a and N-mut cells (Fig. 4*B*). Both of these have high ATPase rates; yet, only the H^+-ATPase of RN1a cells pumps protons efficiently and, hence, gives a high pH^{in} (Fig. 2 *A* and *B*). Furthermore, the glycolytic rates are relatively insensitive to changes in the extracellular and hence, intracellular, pH (Fig. 4*B*). Glycolysis produces 1 ATP and 1 H^+ per lactate. If (*i*) all ATP for the H^+-ATPase is provided by glycolysis, (*ii*) all glycolytic H^+ is excreted by the ATPase, and (*iii*) the stoichiometry of the ATPase is 1 H^+ per ATP, then a futile cycle would result. The higher steady-state pH^{in} of the RN1a cells rules out this possibility; yet, it does not preclude some "futile cycling" from occurring.

In summary, this system exhibits most traits of tumorigenic transformation: tumorigenicity in nude mice, soft agar growth, high-density growth, serum-independent growth, and high rates of aerobic glycolysis. This phenotype is the result of a single transfection with an active plasma-membrane H^+-ATPase. Because the primary effects of this transfection are known, determining the events required for tumorigenic transformation is a tractable problem. This system raises the possibility that H^+-ATPases play a role in the etiology of carcinogenesis.

This work was supported by National Institutes of Health Grant RO1 GM43046-01.

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