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^{31}P NMR Analysis of Intracellular pH of Swiss Mouse 3T3 Cells: Effects of Extracellular Na^+ and K^+ and Mitogenic Stimulation

Mortimer M. Civan†, Stephen R. Williams‡, David G. Gadian‡, and Enrique Rozengurt†
Imperial Cancer Research Fund Laboratories† and the Royal College of Surgeons‡, Lincoln's Inn Fields, London WC2A, England

Summary. Swiss mouse 3T3 cells grown on microcarrier beads were superfused with electrolyte solution during continuous NMR analysis. Conventional ^{31}P and ^{19}F probes of intracellular pH (pH_c) were found to be impracticable. Cells were therefore superfused with 1 to 4 mM 2-deoxyglucose, producing a large intracellular, pH-sensitive signal of 2-deoxyglucose phosphate (2DGP). The intracellular incorporation of 2DGP inhibited the Embden-Meyerhof pathway. However, intracellular ATP was at least in part retained and the cellular responsivity to changes in extracellular ionic composition and to the application of growth factors proved intact. Transient replacement of external Na^+ with choline or K^+ reversibly acidified the intracellular fluids. Quiescent cells and mitogenically stimulated cells displayed the same dependence of shifts in pH_c on external Na^+ concentration (c^o_{Na}). pH_c also depended on intracellular Na^+ concentration (c^c_{Na}). Increasing c^c_{Na} by withdrawing external K^+ (thereby inhibiting the Na,K-pump) caused reversible intracellular acidification; subsequently reducing c^o_{Na} produced a larger acid shift in pH_c than with external K^+ present. Comparison of separate preparations indicated that pH_c was higher in stimulated than in quiescent cells. Transient administration of mitogens also reversibly alkalinized quiescent cells studied continuously. This study documents the feasibility of monitoring pH_c of Swiss mouse 3T3 cells using ^{31}P NMR analysis of 2DGP. The results support the concept of a Na/H antiport operative in these cells, both in quiescence and after mitogenic stimulation. The data document by an independent technique that cytoplasmic alkalinization is an early event in mitogenesis, and that full activity of the Embden-Meyerhof pathway is not required for the expression of this event.

Key Words: 2-deoxyglucose phosphate · inorganic phosphate · ^{19}F NMR · NMR probe configuration · Na/H antiport · vasopressin · insulin · epidermal growth factor · microcarrier beads

Introduction

Cultured fibroblasts have constituted a useful model for the study of mitogenesis [37]. Quiescent cells can be stimulated to reinitiate DNA synthesis by the addition of serum, certain mitogens singly, or a wider range of growth factors in combination [42]. All such combinations which effectively stimulate mitogenesis also increase Na^+ influx into a broad spectrum of quiescent cells, including Swiss mouse 3T3 fibroblasts [43]. The enhanced cellular uptake of Na^+ stimulates the turnover of the Na,K-exchange pump [41, 45] leading to an increase in intracellular K^+ concentration [41, 48], which is associated with the onset of increased DNA synthesis [4, 19].

The concept that enhanced Na^+ entry is an important step in the development of the mitogenic response is supported by four lines of evidence: (i) the apparently constant finding of increased Na^+ influx following addition of mitogens to 3T3 [8, 20, 21, 40, 45] and many other cells [5, 9, 16, 20, 23, 25, 29, 30, 36, 50]; (ii) the synergistic mitogenic response effected by increasing plasma membrane Na^+ permeability together with other mitogenic factors [39, 40]; and (iii) the inhibition of the mitogenic response to serum [45] and to the combinations of epidermal growth factor (EGF) and insulin [38] or EGF, insulin and vasopressin [4] produced by reducing the Na^+ concentration of the culture medium; and (iv) a specific mutation abolishing Na/H antiport activity in hamster fibroblasts precludes growth at neutral and acidic pH in bicarbonate-free medium [34].

The results of a number of published reports have further suggested that the mitogen-stimulated Na^+ influx proceeds through a Na/H antiport, both in Swiss mouse 3T3 cells [4, 44] and in other fibroblasts [24, 31]. In Swiss mouse 3T3 cells, the existence of a Na/H antiport has been suggested by the observations [44] that: (i) addition of Na^+ to Na^+-depleted cells causes intracellular alkalinization, (ii) this effect is cation-specific and inhibitable by amiloride, and (iii) extracellular alkalinization increases the initial rate of Na^+ influx into these cells. As yet, the effect on intracellular pH regulation caused by increasing intracellular Na^+ concentration above physiologic levels has not been reported for Swiss

mouse 3T3 cells. In this respect, the documentation of a Na/H antiport in this widely used model system has been incomplete. It should also be appreciated that the existing documentation of the antiport in Swiss mouse 3T3 cells has been largely based upon estimates of intracellular pH (pH_c) calculated from measurements of the distribution of DMO (5,5-dimethyl oxazolidine-2,4-dione).

In view of these points and the indication that activation of the antiport may be an early universal step in growth regulation, we wished to examine the problem with an entirely independent approach.

In the current work, we have studied Na/H exchange with NMR spectroscopy. As discussed below, conventional ^{31}P and ^{19}F NMR analysis have been found to be inappropriate for studying these cells. However, it has proved possible to monitor intracellular pH continuously by introducing 2-deoxyglucose phosphate (2DGP) into the cells. This maneuver alters the metabolic state of the 3T3 cells, but the responsiveness of the plasma membranes to changes in Na^+ gradient, to external K^+, and to the addition of growth factors is retained. Thus, it has been practicable to study Na/H activity before, during and after experimental perturbations, with each preparation of cells serving as its own series control. The results provide further, complementary evidence supporting the role of a Na/H antiport mechanism in Swiss mouse 3T3 cells, under both quiescent and mitogenically stimulated conditions.

Materials and Methods

Cells

Swiss mouse 3T3 cells [47] were maintained as stock cultures at 37°C in Dulbecco's modified Eagle's medium (DEM) containing 10% fetal calf serum, 100 μg/ml streptomycin, and 100 units/ml penicillin in a humidified atmosphere of 10% CO_2-90% air. Cells were subcultured to 90 mm Nunc petri dishes with medium containing 10% fetal calf serum and grown to confluence. Cells were finally seeded at a final concentration of 5×10^4/ml in a suspension of Cytodex 2 beads (Pharmacia, Uppsala, Sweden) in DEM (750 mg/500 ml). To ensure cell viability, it was important to stir the suspension at about 20 rpm [MCS microcarrier stirrer, Techne (Cambridge) Limited, Duxford, Cambridge, U.K.], just fast enough to prevent the beads from settling. Preparations were usually studied 5 to 8 days later. When cells were to be analyzed in the quiescent state, the concentration of serum was usually lowered to 1% the day before harvesting.

DNA synthesis was assayed in a 1 : 1 mixture of DME and Waymouth medium [22], containing 1 μCi/ml (3-H) thymidine (Radiochemical Centre, Amersham, U.K.) and a total thymidine concentration of 1 μM, with or without growth factors. The incorporation of radioactivity into trichloroacetic acid-insoluble material was measured after incubation for 20 to 26 hr. In six preparations so studied, growth factors increased tritiated thymidine incorporation 18 ± 6-fold (mean ± SE).

Perfusion

The cell-covered beads were transferred to an analysis chamber with plastic pipettes, and washed free of incubation medium with an electrolyte solution (ES) consisting of (in mM): NaCl, 140; KCl, 5; $CaCl_2$, 1.8; $MgCl_2$, 0.9; Na_2HPO_4, 0.7; NaH_2PO_4, 0.3; glucose, 25; HEPES, 15; Tris base, 6.5; and glutamine, 2.5; at a pH of 7.1 U and at 37°C. The chamber had an inner diameter of 16 mm and a sintered glass disc for supporting the cells of pore size to 41 to 100 μm (Corning, Stafforshire, U.K.). The beads were loaded to a height of some 30 mm.

NMR Analysis

The ^{31}P NMR studies were carried out at 145.7 MHz with a Bruker AM360WB spectrometer, using a vertical 8.5 T magnet and a home-built probe. In order to optimize sensitivity [14], we considered a solenoidal radiofrequency coil as used, e.g., by Ugurbil et al. [49] rather than a saddle-shaped coil. However, experimental comparisons of the performance of saddle-shaped and solenoidal coils showed only a modest (~1.3-fold) improvement in sensitivity with the solenoidal coil, primarily because of resistive losses associated with the sample [10, 11, 13]. This factor of 1.3 was more than offset by the degraded resolution and the difficulty in using a horizontal sample chamber. Therefore, the probe incorporated a saddle-shaped radiofrequency coil into which the chamber was placed. The cells were continuously superfused at 1.3 ml/min with a peristaltic pump during the NMR studies. The coil was tuned with the sample in place. The magnetic field homogeneity was then optimized using the water proton signal. The value of the field was adjusted so that the water proton signal was on resonance at the same absolute frequency for all experiments, providing an effective reference standard [1]. A proton line width of 30 Hz was routinely attained. Pulses were applied with a tip angle of 25° at an interpulse interval of 144 msec. Spectra were usually generated by Fourier transformation of 2,000 to 5,000 transients accumulated over 5 to 12 min.

Results

Conventional ^{31}P and ^{19}F NMR Analysis

Endogenous intracellular inorganic phosphate (P_i) provides an ideal NMR index of intracellular pH [26] since no foreign marker need be introduced into the cell. However, this now conventional approach proved impracticable with the Swiss mouse 3T3 cells. Although the ATP signals were clearly visible (Fig. 1), the endogeneous P_i signal was too small to serve as a reliable parameter of intracellular pH (pH_c) when the cells were superfused with a solution lacking P_i.

Cells with a poorly detectable P_i signal can be loaded with esters of nontoxic fluorinated amino acids; substantial intracellular concentrations of free fluorinated amino acids can result if the cells possess sufficient endogenous esterase activity and if the amino acids cannot escape across the plasma

membranes [46]. In some tissues, both approaches can be applied in a complementary fashion [6, 18]. This approach, too, proved unsatisfactory with the cells used. Incubation of quiescent cells with α-(difluoromethyl)-alanine methyl ester for 18 hr did not result in a detectable NMR signal of the free fluorinated methyl alanine. The quiescent cells either did not take up or did not cleave the methyl ester. Incubation of cells stimulated with growth factors did produce substantial de-esterification of the fluorinated probe. However, at least at 37°C, the mitogenically stimulated cells released all of the detectable free amino acid to the external medium.

^{31}P NMR of 2DGP

These technical difficulties were addressed by using 2-deoxyglucose phosphate as an alternative probe of intracellular pH [2, 49], as originally suggested by Navon et al. [27]. 2-Deoxyglucose has been used as an investigation tool to block the Embden-Meyerhof pathway. In the present study, perfusion with inadequate oxygenation caused 3T3 cell damage. However, in the presence of satisfactory oxygen and substrate delivery, the cells retained ATP at least in part (Fig. 7), presented a stable ^{31}P spectrum, and responded reversibly to the experimental perturbations applied. 2-Deoxyglucose has also been reported to cause little change in the cells of perfused hearts, when care is exercised to provide adequate oxygenation and substrate [2]; the incorporation of large concentrations of 2DGP produces an acid shift of only 0.06 U.

In the present work, the cell-coated beads were incubated at 37°C with a well-oxygenated, glucose-free solution containing 1 to 4 mM 2-deoxyglucose, 1 mM pyruvate, 5 mM glutamine and 1 mM P_i. Analysis of the signals from external P_i and internal 2DGP permitted simultaneous monitoring of external (pH_o) and intracellular pH, respectively. The spectral position of 2DGP was noted to be satisfactorily stable under baseline conditions for periods as long as 6 to 7 hr, as documented below (Fig. 8 legend).

Calibration Curves of Chemical Shifts as Functions of pH

The chemical shifts of P_i and 2DGP were measured [relative to phosphocreatine (PCr) at neutral pH] in ppm using a program of the Aspect 3000 computer. Values of pH_c and pH_o were calculated from empirical equations fitting the calibration curves obtained with simple aqueous solutions at 37°C (Fig. 2):

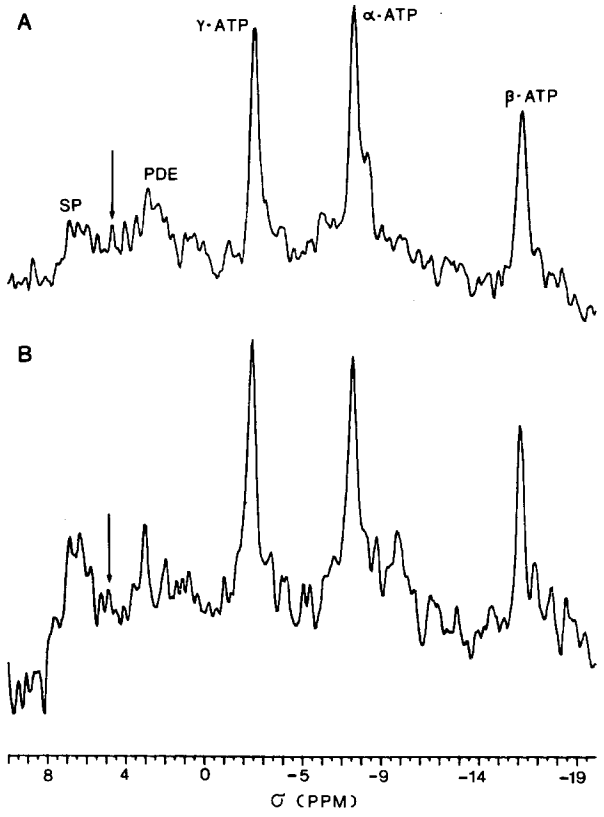

Fig. 1. ^{31}P NMR spectrum of Swiss mouse 3T3 cells superfused with electrolyte solution free of inorganic phosphate and 2-deoxyglucose. (*A*) Spectrum obtained after accumulating 165,000 transients before the addition of growth factors. (*B*) Spectrum generated from 120,000 transients after the addition of growth factors to the superfusing solution. Despite the excellent signal-to-noise ratio characterizing the ATP signals, the intracellular inorganic phosphate signal cannot be distinguished from the background noise; the arrows indicate the presumed positions of the P_i signals

$$pH_c = 6.23 + \log[(\sigma_{2DGP} - \sigma_{PCr} - 3.74)/(7.58 - \sigma_{2DGP} + \sigma_{PCr})] \quad (1)$$

$$pH_o = 6.75 + \log[(\sigma_{Pi} - \sigma_{PCr} - 3.06)/(5.72 - \sigma_{Pi} + \sigma_{PCr})]. \quad (2)$$

An approximate estimate of the precision of a single pH measurement is provided by considering the eight preparations studied in the quiescent state in the present work. As discussed below, the 2DGP signal is substantially smaller in quiescent than in stimulated cells. The standard deviation of the measured values of pH_c for the samples was 0.038 pH units; this value is an upper bound to the actual instrumental uncertainty, reflecting both the instrumental and biological variance from preparation to preparation.

The accuracy of the technique is approximately 0.1 pH unit, 2 to 3 times poorer than the precision, as illustrated by Fig. 2. Identical calibration curves

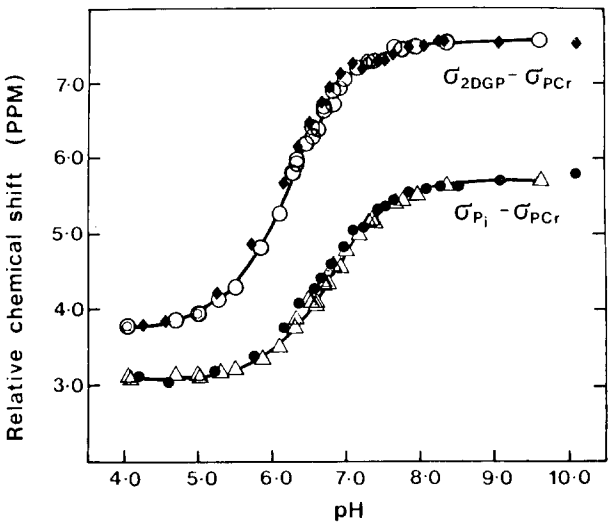

Fig. 2. Chemical shifts of 2DGP and P_i (relative to PCr at neutral pH) as functions of pH in aqueous solution. The analyses were conducted with a low Na-high K solution (closed symbols) six weeks after spectral measurements of a high Na-low K solution (open symbols). The slight displacements of the open and closed symbols from each other provide an estimate of the uncertainty associated with the calibration procedure. The low Na-high K solution contained (in mM): KCl, 110; K_2HPO_4, 5.2; HEPES, 10; MES, 10; Na_2-2DGP, 5; and Na_2PCr, 5. The high Na-low K solution was similar to the standard electrolyte solution except for the additions of 5 mM Na_2-2DGP and 5 mM Na_2PCr, and the replacements of sodium phosphate by 5 mM Tris $KHPO_4$ and of glucose and glutamine by 1 mM Tris pyruvate

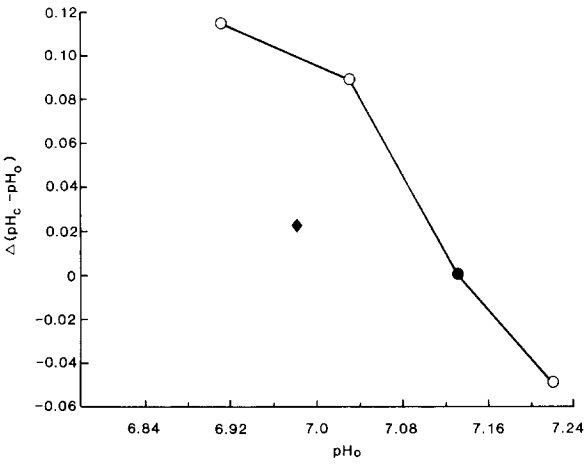

Fig. 3. Transmembrane pH as a function of external pH. The change in transmembrane pH gradient [$\Delta(pH_c - pH_o)$] relative to its value under reference conditions [142 mM Na^+, pH_c = 6.82 U, pH_o = 7.13 U, ($pH_c - pH_o$ = −0.28 U), filled circle] has been plotted against pH_o. The rhomboid was obtained with choline medium; all other points were measured during superfusion of the same preparation with Na^+ electrolyte solution. In each case, pH_o has been calculated from the observed value of σ_{P_i} using Eq. (2)

have been obtained for P_i as a function of pH in the presence of high Na-low K and of low Na-high K solutions, both at 25°C [11] and at 37°C (noted in the present study). However, the dependence of the chemical shift for P_i as a function of pH can vary slightly. The magnitude of this slight variation is illustrated by Fig. 2. The small displacements of the two calibration curves obtained at different times, for both 2DGP and P_i, in high and low Na-containing media are considered to reflect the variance of the procedure, rather than demonstrating a significant dependence upon the ionic composition of the aqueous solution. Although the calibration curve for P_i appears independent of the ratio of Na^+ and K^+ concentrations, it is affected by the presence of external choline (*as noted below*).

Na/H Exchange in Stimulated Cells

Basal Values of Intracellular pH and Transmembrane pH Gradient

In 14 preparations superfused with vasopressin (20 ng/ml), insulin (1 μg/ml) and epidermal growth factor (5 ng/ml), the intracellular pH was 6.90 ± 0.02 U in the presence of an extracellular pH of 7.15 ± 0.01 U. These and subsequent averaged values are presented as means ± SE. The paired transmembrane difference in pH, ($pH_c - pH_o$), was −0.25 ± 0.03 U; this value is algebraically much larger than the range of equilibrium values of −0.6 to −0.9 U expected for a cell with a membrane potential of −35 to −55 mV [15, 17, 32, 36, 51]. Figure 3 (circles) illustrates the effect of changes in external pH on this transmembrane difference in pH. Changes in pH_o over the range 6.92 to 7.22 U produced relatively smaller changes in pH_i, so that the transmembrane difference in pH was appreciably altered.

Effects of Replacing External Na^+

Replacement of extracellular Na^+ by choline produced a reversible acid shift (upfield, to the right in Fig. 4) in the 2DGP signal. In each of 13 experiments in which c^o_{Na} was reduced from 142 to 0-1.7 mM, pH_c fell; the mean ± SE was −0.18 ± 0.02. In the representative sample of Fig. 4, the serial removal and replacement of extracellular Na^+ was performed three times; in each case, an intracellular acidification was produced of approximately constant magnitude (−0.24 to −0.26 pH U).

In Fig. 4, each change in superperfusing solution was associated with an upfield shift in the P_i

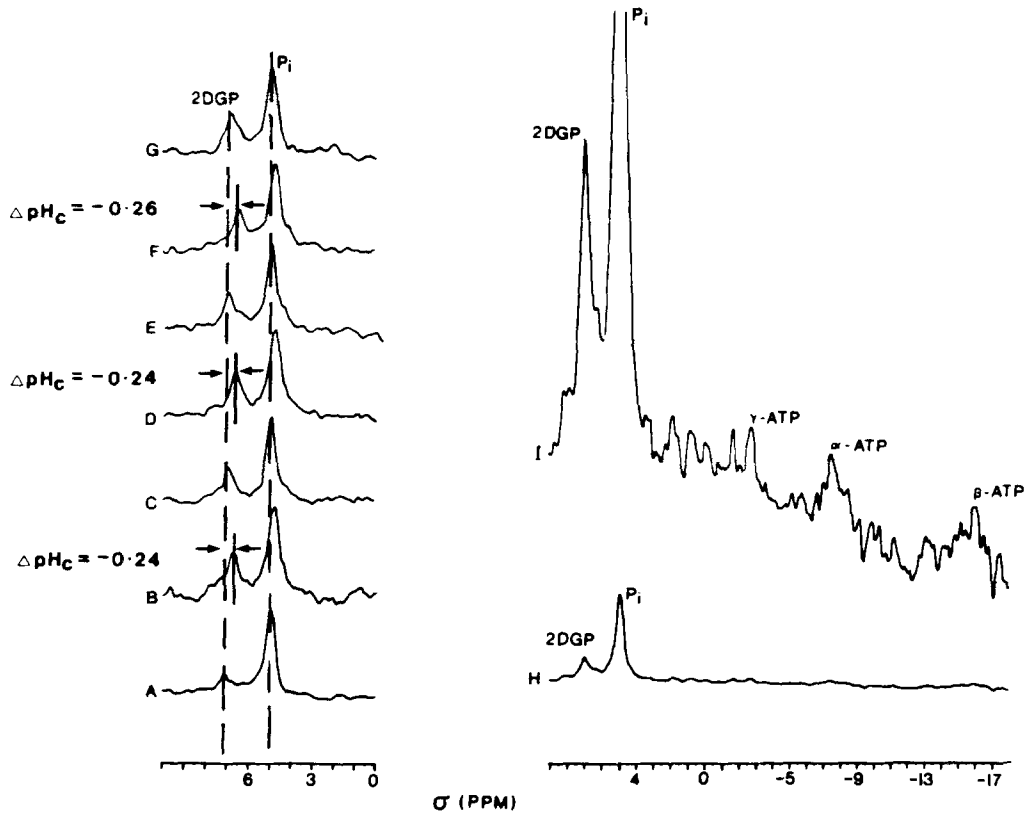

Fig. 4. Effect of sequential and complete replacement of external Na^+ by choline in the presence of growth factors. Spectra *A*, *C*, *E*, *G*, *H* and *I* were obtained with the cells superfused with Na-containing electrolyte solution, while spectra *B*, *D*, and *F* were collected during periods of superfusion of the same preparation with choline solution. Figures *H* and *I* present the same spectrum obtained from 30,000 scans, but at different magnifications, and show the relative sizes of the 2DGP and ATP signals in this sample. The values for pH and NS of spectra *A-G* were: (*A*) 7.08 U, 15,000 scans; (*B*) 6.77 U, 4,451; (*C*) 6.97 U, 5,000; (*D*) 6.72 U, 5,000; (*E*) 6.94 U, 5,000 (*F*) 6.64 U, 3,860; and (*G*) 6.82 U, 5,000

signal. It should be pointed out that the pK_a of the phosphate buffer was shifted by 0.18 U when the Na^+ of the external electrolyte solutions was largely replaced by choline[1]. When this is taken into account, it is clear that the fall in pH_c was not a consequence of the shift in σ_{Pi} associated with choline replacement, and that the external pH was not actually displaced. In addition, even if the shift in pK_a is not taken into account, the magnitude of the intracellular acidification was much larger than could be attributed to the apparent small acidification of the external medium (Fig. 3, rhomboid).

As illustrated by Fig. 5, the equimolar substitution of K^+ for Na^+ also led to an intracellular acidification; for the three substitutions conducted in two preparations, ΔpH_c was -0.090 ± 0.007 U.

[1] In contrast, replacement of choline for K^+ in a 120-mM KCl solution buffered with 20 mM HEPES to a pH of 6.9 U at 37°C did not shift the 2DGP signal. Therefore, the intracellular acid shifts of 2DGP noted after choline substitution for external Na^+ cannot be ascribed to a putative cellular uptake of choline.

The dependence of intracellular pH on external sodium concentration is a graded effect. A half-maximal response was observed when c^o_{Na} was approximately 35 mM (Fig. 6, circles).

Effects of Removing External K^+

The rate of turnover of a Na/H antiport should depend not only on external, but also on intracellular Na^+ (c^c_{Na}) concentration. Two results of the present study suggest that the pH_c of Swiss mouse 3T3 cells does depend on c^c_{Na}. Both observations were made with three preparations whose Na,K-exchange pumps were inhibited by removing extracellular K^+. First, the reduced rate of Na^+ extrusion should have led to a rise in c^c_{Na}, decreasing the chemical gradient favoring Na^+ entry, and thereby slowing the rate of ejection of protons. An acid shift in intracellular pH was, in fact, observed in each sample; ΔpH was -0.09 ± 0.01 U. Second, under these conditions of pump inhibition, the dependence of pH_c

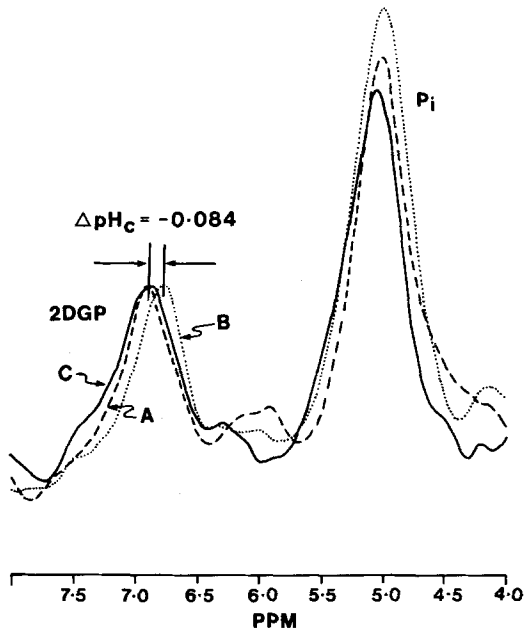

Fig. 5. Effect of K^+ substitution for Na^+ in external bathing medium. All three spectra were obtained with same sample of cell-coated beads. (*A*) Initial superfusion with sodium-containing electrolyte solution; NS = 4,500 scans and pH_c = 6.91 U. (*B*) Superfusion with similar solution except for the equimolar replacement of Na^+ by K^+; NS = 4,915 scans and pH_c = 6.81 U. (*C*) Return to superfusion with the standard sodium-containing saline solution; NS = 2,742 scans and pH_c = 6.88 U

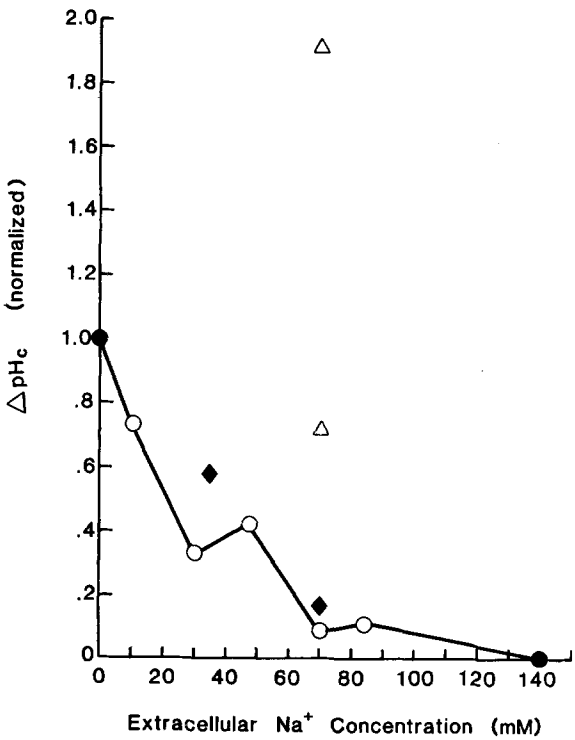

Fig. 6. Fractional change in intracellular pH as a function of extracellular Na^+ concentration (c^o_{Na}). The reference pH is the value of pH_c at an extracellular Na^+ concentration of approximately 140 mM. The maximal change in pH_c [$(\Delta pH_c)_{max}$] is that shift in intracellular pH observed when c^o_{Na} was reduced to 0 to 1.7 mM. The changes in pH_c noted at other values of c^o_{Na} (ΔpH_c) have been normalized by dividing the observed values by the corresponding value of $(\Delta pH_c)_{max}$ measured in the same preparation. Measurements were obtained in the quiescent state (filled rhomboids, two preparations), and during mitogenic stimulation in the presence (open circles, four preparations) and absence (open triangles, two preparations) of external K^+.

on c^o_{Na} was changed. In contrast to the behavior noted under baseline conditions (Fig. 6), reducing the external Na^+ concentration to 70 mM now lowered intracellular pH by 0.08 and 0.22 U in two different samples (Fig. 6, triangles).

Na/H Exchange in Quiescent Cells

In the quiescent state, the baseline values for pH_c, pH_o and ($pH_c - pH_o$) were 6.76 ± 0.01, 7.11 ± 0.01 and −0.35 ± 0.02 U, respectively, based on eight preparations. The mean values of intracellular pH measured with quiescent (6.76 ± 0.01 U) and stimulated cells (6.90 ± 0.02 U) were different; this difference was 0.14 ± 0.03 U (standard deviation of the difference in the means, $P < 0.001$ using Student's *t*-test).

Although quiescence was associated with a lower absolute baseline of intracellular pH, the fractional change in pH_c measured in two preparations as a function of external Na^+ concentration was approximately the same as in mitogenically stimulated cells (Fig. 6, rhomboids).

In view of the observed difference in steady-state values of pH_c in quiescent and stimulated cells, it was of great interest to determine whether the addition of the mitogenic combination of growth factors to a quiescent population of Swiss mouse 3T3 cells could acutely induce cytoplasmic alkalinization.

Effects of Adding Growth Factors to Quiescent Cells

The combination of mitogens used in the present study was vasopressin (20 ng/ml), insulin (1 μg/ml) and epidermal growth factor (5 ng/ml); together, these pure peptide mitogens stimulate DNA synthesis almost as much as does serum [6, 17]. In the experiment of Fig. 7, the growth factors reversibly alkalinized the cells by 0.17 U; the mean ± SE for the change in pH_c measured with three preparations was 0.12 ± 0.02 U.

The current superfusion system permitted the assessment of repeated transient administrations of mitogens. In the experiment of Fig. 8, the same

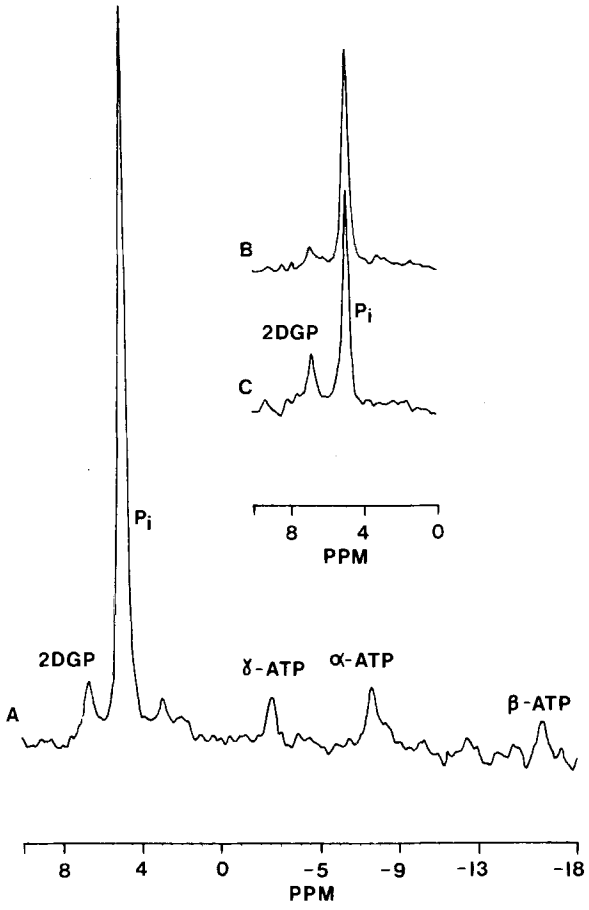

Fig. 7. Effects of growth factors on ^{31}P spectrum from quiescent cells. (*A*) Initially, the concentrations of 2DGP and ATP appear to be of similar magnitude; pH_c = 6.73 U, NS = 54,152. (*B*) Directly after superfusing the same beads with mitogens, the intracellular pH shifted to 6.90 U; NS = 15,000. (*C*) Following the alkaline shift, the 2DGP signal increased severalfold while pH_c relaxed to 6.84 U; NS = 10,000

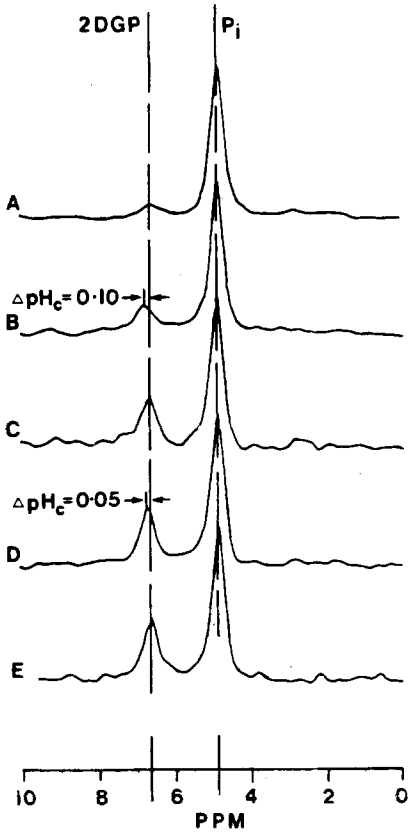

Fig. 8. Effects of transient superfusion of growth factors on 2DGP signal. Spectra *A*, *C* and *E* were collected in the absence of growth factors, while spectra *B* and *D* were obtained during the transient administration of vasopressin, insulin and epidermal growth factor. The values of pH_c and NS for each of the spectra were: (*A*) 6.73 U, 54,152 scans; (*B*) 6.84 U, 29,848; (*C*) 6.74 U, 9,570; (*D*) 6.78 U, 24,747; and (*E*) 6.73 U, 11,976. Spectra *A*, *C* and *E* document the stability of the spectral frequency of 2DGP under the same reference conditions; over a period of 6.5 hr, σ_{2DGP} was unchanged within a range of 0.03 ppm (equivalent to 0.02 pH U)

cells were superfused with electrolyte solutions alternately containing and free of mitogens. The two additions of growth factors produced reversible alkalinizations of 0.10 and 0.05 U.

An additional, later effect of the mitogens is illustrated by Figs. 7*B-C* and 8*A-C*. Following the initial alkalinization, the size of the 2DGP signal increased severalfold in both preparations. Of particular interest was the observation that the values of pH_c were the same in Figs. 8*A* (before adding growth factors) and 8*C* (after washing them off). Thus, under the same conditions, pH_c was unchanged, despite the much larger concentration of 2DGP in Fig. 8*C* than in Fig. 8*A*. Apparently, 2DGP does not substantially change the intracellular pH of these cells, in agreement with the observation that only a small acid shift of 0.06 U was observed in perfused hearts [2].

Discussion

The results of the present study document the feasibility of monitoring the intracellular pH of Swiss mouse 3T3 cells grown on microcarrier beads by ^{31}P NMR analysis of 2-deoxyglucose phosphate (2DGP). A similar capability has been reported for mouse embryo fibroblasts (C3H/10T1/2) based primarily on monitoring intracellular P_i, but also by studying 2DGP [49]. In that study, stress was placed on the enhanced instrumental sensitivity resulting from use of a solenoid, rather than a more conventional saddle-shaped coil. However, the theoretical and experimental considerations presented in the Materials and Methods section indicate that a saddle-shaped coil provides close to optimal detecting capability for the current samples.

The current results have also demonstrated that, contrary to expectation, conventional ^{31}P NMR analysis of intracellular inorganic phosphate and ^{19}F analysis of intracellular α-(difluoromethyl)-alanine are inapplicable for monitoring pH_c in these cells (at least at 37°C). Instead, 2DGP has been used as an alternative probe of intracellular pH. 2-Deoxyglucose inhibits the glycolytic pathway in 3T3 cells, which may interfere with the full mitogenic response to growth factors. However, with attention to adequate oxygen and substrate delivery, intracellular ATP signals were clearly visible in the presence of 2DGP (Fig. 7). Furthermore, the data indicate that the integrity of the membrane function is intact. That 2DGP is, in fact, satisfactory for monitoring pH_c has been documented by three observations: (i) under favorable conditions, the chemical shift of 2DGP is stable for periods as long as 6 to 7 hr (Fig. 8), (ii) the accumulation of 2DGP within the cells does not appear to change the baseline chemical shift greatly (Fig. 8), and (iii) reversible changes in σ_{2DGP} can be produced both by inhibiting Na^+ transport (either by removing external Na^+ or by inhibiting the Na,K-exchange pump) and by mitogenically stimulating the cells. There can be little doubt that the superfused cells, anchored to the Cytodex beads, do respond to growth factors in the presence of 2-deoxyglucose; in the current work, the mitogens stimulated an increase in endogenous esterase activity, an alkalinization of the intracellular fluids, and an increase in the rate of deoxyglucose entry and/or phosphorylation.

The present results, based on ^{31}P NMR analysis of intracellular 2DGP have provided further evidence for the operation of a Na/H antiport in the plasma membranes of Swiss mouse 3T3 cells. Measurements of the distribution of labeled DMO have suggested that the addition of external Na^+ to Na^+-depleted cells causes intracellular alkalinization [44]. In the current study, transient reduction of the extracellular Na^+ concentration (c^o_{Na}) has produced a graded intracellular acidosis. The half-maximal effect was observed at identical concentrations of external Na^+, 30 to 40 mM. In fact, when plotted using the same convention, Fig. 2*B* of the earlier study [44] is nearly congruent with the present Fig. 6, expressing the relationship between the magnitude of the acid shift as a function of c^o_{Na}. Schuldiner and Rozengurt [44] also reported that alkalinizing the external medium stimulated Na^+ uptake by 3T3 cells.

It is of interest that intracellular acidification is observed when Na^+ is replaced either by choline or K^+. Choline substitution would be expected to halt charge dissipation through whatever Na^+ conductance channels are present, hyperpolarizing the membrane; K^+ substitution would depolarize the membrane, as in other cells [e.g., Ref. 7]. The fact that both ionic substitutions produced acidification suggests that the underlying mechanism is more likely to be an electroneutral than a rheogenic process.

In the present study, we report the additional observation that inhibition of the Na,K-exchange pump (by removing external K^+) causes reversible acidification of the intracellular fluids. Furthermore, after removal of extracellular K^+, subsequent reduction of the external Na^+ concentration markedly increases the ensuing intracellular acidification (Fig. 6). The current results strongly indicate that pH_c depends not only on external, but also on internal Na^+ concentration. This demonstration that pH_c is partly regulated by the gradient in Na^+ concentration across the plasma membrane is a necessary condition for documenting the operation of a Na/H antiport mechanism.

The present data provide additional support for the concept that mitogens alkalinize the intracellular fluids of these [3, 44] and other fibroblasts [5, 24, 29, 31]. The results further demonstrate that full functioning of the Embden-Meyerhof pathway is not required for the very early steps in the mitogenic response to take place. Comparison of separate populations of quiescent and mitogenically stimulated preparations suggested that the growth factors alkalinized the 3T3 cells by 0.14 ± 0.03 U. The magnitude of this change is comparable to the range of estimates of 0.15 to 0.21 reported following incubation of Swiss mouse 3T3 cells with serum [3] or combinations of mitogens [3, 4, 44]; the baseline intracellular pH of the quiescent cells (6.76 ± 0.01) also falls in the range of estimates (6.56 ± 0.03 [Table 2, Ref. 4] to 7.21 ± 0.02 [44]) calculated from DMO distributions in these cells, when the external pH was 7.0 U. The mitogen-induced alkalinization was further and more strongly documented in the present work by monitoring pH_c of single preparations of cells before, during and after the transient administration of growth factors. In the sample of Fig. 8, a second transient addition of the mitogens produced a second, albeit smaller, intracellular alkalinization.

^{31}P NMR analysis of 2-deoxyglucose phosphate provides a novel approach for studying Na/H exchange by fibroblasts. It complements the other techniques already applied to the problem: measurement of DMO distribution [44] and fluorescence analysis of intracellular probes [5, 24]. Of these approaches, the DMO technique has the advantage of simplicity, facilitating rapid analysis of many preparations under a range of experimental conditions. However, unlike NMR and fluorescence analyses,

DMO measurements do not allow the use of a single sample as its own series control. Fluorescence study of pH-sensitive probes is far more sensitive than ^{31}P NMR spectroscopy, leading to better time resolution. However, leakage of the uncoupled intracellular fluorescent probe to the bathing medium as well as other serious disadvantages can limit the applicability of the technique, especially at 37°C [12]. For example, based on the data of Moolenaar et al. [24], only about 20% of the initial content of the dye indicator BCECF would have been expected to have remained intracellular (even at 30°C) by the end of the experiment of Fig. 8; thus, conducting prolonged experiments at 37°C becomes problematic using fluorescent spectroscopy. This problem can be circumvented by coupling dextran to a fluorescent probe [5, 35]; however, loading of the dye requires osmotic lysis of pinocytotic vesicles, which may also alter the state of some cells. The advantages of ^{31}P NMR analysis of 2DGP in fibroblasts are: the prolonged stability of the spectrum, the capability of studying a single sample both under experimental and control conditions, and the opportunity to monitor other intracellular signals simultaneously. The ATP signal can be simply monitored from the same ^{31}P spectrum (Fig. 7); in principle, the intracellular contents of 23Na and 39K could also be monitored within the same sample, using double coils and external shift reagents [12*a*, 28, 33]. The current study demonstrates the feasibility of the ^{31}P NMR approach for continuously monitoring intracellular pH and for examining Na/H exchange by quiescent and mitogenically stimulated cells.

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References

1. Ackerman, J.J.H., Gadian, D.G., Radda, G.K., Wong, G.G. 1981. Observations of ^{1}H NMR signals with receiver coils tuned for other nuclides. *J. Magn. Reson.* **42:**498–500
2. Bailey, I.A., Williams, S.R., Radda, G.K., Gadian, D.G. 1981. Activity of phosphorylase in total global ischaemia in the rat heart: A phosphorus-31 nuclear-magnetic-resonance study. *Biochem. J.* **196:**171–178
3. Burns, C.P., Rozengurt, E. 1983. Serum, platelet-derived growth factor, vasopressin and phorbol esters increase intracellular pH in Swiss mouse 3T3 cells. *Biochem. Biophys. Res. Commun.* **116:**931–938
4. Burns, C.P., Rozengurt, E. 1984. Extracellular Na^+ and initiation of DNA synthesis: Role of intracellular pH and K^+. *J. Cell. Biol.* **98:**1082–1089
5. Cassel, D., Rothenberg, P., Zhuang, Y.X., Deuel, T.F., Glaser, L. 1983. Platelet-derived growth factor stimulates Na^+/H^+ exchange and induces cytoplasmic alkalinization in NR6 cells. *Proc. Natl. Acad. Sci. USA* **80:**6224–6228
6. Civan, M.M., Lin, L.E., Peterson-Yantorno, K., Taylor, J., Deutsch, C. 1984. Intracellular pH of perfused single frog skin: Combined ^{19}F- and ^{31}P-NMR analysis. *Am. J. Physiol.* **16:**C506–C510
7. DeLong, J., Civan, M.M. 1984. Apical sodium entry in split frog skin: Current-voltage relationship. *J. Membrane Biol.* **82:**25–40
8. Dicker, P, Rozengurt, E. 1980. Phorbol esters and vasopressin stimulate DNA synthesis by a common mechanism. *Nature (London)* **287:**607–612
9. Felber, S.M., Brand, M.D. 1983. Concanavalin A causes an increase in sodium permeability and intracellular sodium content of pig lymphocytes. *Biochem. J.* **210:**893–897
10. Gadian, D.G., 1982. Nuclear Magnetic Resonance and its Applications to Living Systems. Clarendon, Oxford
11. Gadian, D.G., Robinson, F.N.H. 1979. Radiofrequency losses in NMR experiments on electrically conducting samples. *J. Magn. Reson.* **34:**449–455
12. Graber, M.L., DiLillo, D.C., Friedman, B.L., Pastoriza-Munoz, E. 1986. Characteristics of fluoroprobes for measuring intracellular pH. *Anal. Biochem.* **156:**202–212

12*a*. Gupta, R.K., Gupta, P. 1982. Direct observation of resolved resonances from intra- and extracellular sodium-23 ions in NMR studies of intact cells and tissues using dysprosium (ITT) tripolyphosphate as paramagnetic shift reagent. *J. Magn. Reson.* **47:**344–350

13. Hoult, D.I., Lauterbur, P.C. 1979. The sensitivity of the zeugmatographic experiment involving human samples. *J. Magn. Reson.* **34:**425–433
14. Hoult, D.I., Richards, R.E. 1976. The signal-to-noise ratio of the nuclear magnetic resonance equipment. *J. Magn. Reson.* **24:**71–85
15. Kimhi, Y., Palfrey, C., Spector, I., Barak, Y., Littauer, U.Z. 1976. Maturation of neuroblastoma cells in the presence of dimethylsulfoxide. *Proc. Natl. Acad. Sci. USA* **73:**462–466
16. Koch, K.S., Leffert, H.L. 1979. Increased sodium ion influx is necessary to initiate rat hepatocyte proliferation. *Cell* **18:**155–163
17. Lichtshtein, D., Kaback, H.R., Blume, A.J. 1979. Use of a lipophilic cation for determination of membrane potential in neuroblastoma-glioma hybrid cell suspensions. *Proc. Natl. Acad. Sci. USA* **76:**650–654
18. Lin, L.E., Shporer, M., Civan, M.M. 1985. ^{31}P-nuclear magnetic resonance analysis of perfused single frog skins. *Am. J. Phsyiol.* **248:**C177–C180
19. Lopez-Rivas, A., Adelberg, E.A., Rozengurt, E. 1982. Intracellular K^+ and the mitogenic response of 3T3 cells to peptide factors in serum-free medium. *Proc. Natl. Acad. Sci. USA* **79:**6275–6279
20. Mendoza, S.A., Wigglesworth, N.M., Pohjanpelto, P., Rozengurt, E. 1980. Na entry and Na-K pump activity in murine, hamster, and human cells. Effect of monensin, serum, platelet extract and viral transformation. *J. Cell. Physiol.* **103:**17–27
21. Mendoza, S.A., Wigglesworth, N.M., Rozengurt, E. 1980. Vasopressin rapidly stimulates Na^+ entry and Na-K pump

activity in quiescent cultures of mouse 3T3 cells. *J. Cell. Physiol.* **105:**153–162
22. Mierjewski, K., Rozengurt, E. 1977. Vitamin B_{12} enhances the stimulation of DNA synthesis by serum in resting cultures of 3T6 cells. *Exp. Cell Res.* **106:**394–396
23. Moolenaar, W.H., Boonstra, J., Saag, P.T., van der, Laat, S.W. de. 1981. Sodium/proton exchange in mouse neuroblastoma cells. *J. Biol. Chem.* **256:**12883–12887
24. Moolenaar, W.H., Tertoolen, L.G.J., Laat, S.W. de. 1984. The regulation of cytoplasmic pH in human fibroblasts. *J. Biol. Chem.* **259:**7563–7569
25. Moolenaar, W.H., Yarden, Y., Laat, S.W. de, Schlessinger, J. 1982. Epidermal growth factor induces electrically silent Na^+ influx in human fibroblasts. *J. Biol. Chem.* **257:**8502–8506
26. Moon, R.B., Richards, J.H. 1973. Determination of intracellular pH by ^{31}P magnetic resonance. *J. Biol. Chem.* **248:**7276–7278
27. Navon, G., Ogawa, S., Shulman, R.G., Yamane, T. 1977. ^{31}P Nuclear magnetic resonance studies of Ehrlich ascites tumor cells. *Proc. Natl. Acad. Sci. USA* **74:**87–91
28. Ogino, T., Shulman, G.I., Avison, M.J., Gullans, S.R., Hollander, J.A. den, Shulman, R.G. 1985. ^{23}Na and ^{39}K NMR studies of ion transport in human erythrocytes. *Proc. Natl. Acad. Sci. USA* **82:**1099–1103
29. Owen, N.E., Villereal, M.L. 1983. Lys-bradykinin stimulates Na^+ influx and DNA synthesis in cultured human fibroblasts. *Cell* **32:**979–985
30. Paris, S., Pouysségur, J. 1983. Biochemical characterization of the amiloride-sensitive Na^+/H^+ antiport in Chinese hamster lung fibroblasts. *J. Biol. Chem.* **258:**3503–3508
31. Paris, S., Pouysségur, J. 1984. Growth factors activate the Na^+/H^+ antiporter in quiescent fibroblasts by increasing its affinity for intracellular H^+. *J. Biol. Chem.* **259:**10989–10994
32. Philo, R.D., Eddy, A.A. 1978. The membrane potential of mouse ascites-tumor cells studied with the fluorescent probe 3,3′-dipropyloxadicarbocyanine: Amplitude of the depolarization caused by amino acids. *Biochem. J.* **174:**801–810
33. Pike, M.M., Springer, C.S., Jr. 1982. Aqueous shift reagents for high-resolution cationic nuclear magnetic resonance. *J. Magn. Reson.* **46:**348–353
34. Pouysségur, J., Sardet, C., Franchi, A., L'Allemain, G., Paris, S. 1984. A specific mutation abolishing Na^+/H^+ antiport activity in hamster fibroblasts precludes growth at neutral and acidic pH. *Proc. Natl. Acad. Sci. USA* **81:**4833–4837
35. Rothenberg, P., Glaser, L., Schlesinger, P., Cassel, D. 1983. Epidermal growth factor stimulates amiloride-sensitive $^{22}Na^+$ uptake in A431 cells: Evidence for Na^+/H^+ exchange. *J. Biol. Chem.* **258:**4883–4889
36. Rothenberg, P., Reuss, L., Glaser, L. 1982. Serum and epidermal growth factor transiently depolarize quiescent BSC-1 epithelial cells. *Proc. Natl. Acad. Sci. USA* **79:**7783–7787
37. Rozengurt, E. 1980. Stimulation of DNA synthesis in quiescent cultured cells: Exogenous agents, internal signals and early events. *Curr. Top. Cell. Regul.* **17:**59–88
38. Rozengurt, E. 1981. Stimulation of Na influx, Na-K pump activity and DNA synthesis in quiescent cultured cells. *Adv. Enzyme Regul.* **19:**61–85
39. Rozengurt, E. 1981. Na influx as a mitogenic signal for cultured fibroblastic cells. *In*: Cellular Responses to Molecular Modulators. L.W. Mozes, J. Schultz, W.A. Scott, and R. Werner, editors. pp. 149–168. Academic Press, New York
40. Rozengurt, E., Gelehrter, T.D., Legg, A., Pettican, P. 1981. Mellitin stimulates Na entry, Na-K pump activity and DNA synthesis in quiescent cultures of mouse cells. *Cell* **23:**781–788
41. Rozengurt, E., Heppel, L.A. 1975. Serum rapidly stimulates ouabain-sensitive $^{86}Rb^+$ influx in quiescent 3T3 cells. *Proc. Natl. Acad. Sci. USA* **72:**4492–4495
42. Rozengurt, E., Mendoza, S.A. Synergistic signals in mitogenesis: Role of ion fluxes, cyclic nucleotides and protein kinase C in Swiss mouse 3T3 cells. *J. Cell Sci. (in press)*
43. Rozengurt, E., Mendoza, S.A. 1986. Early stimulation of Na^+/K^+ pump activity and Ca^{2+} fluxes in fibroblast mitogenesis. *Curr. Top. Membr. Transp. (in press)*
44. Schuldiner, S., Rozengurt, E. 1982. Na^+/H^+ antiport in Swiss 3T3 cells: Mitogenic stimulation leads to cytoplasmic alkalinization *Proc. Natl. Acad. Sci. USA* **79:**7778–7782
45. Smith, J.B., Rozengurt, E. 1978. Serum stimulates the Na^+,K^+ pump in quiescent fibroblasts by increasing Na^+ entry. *Proc. Natl. Acad. Sci. USA* **75:**5560–5564
46. Taylor, J., Deutsch, C. 1983. Fluorinated α-methylamino acids as ^{19}F indicators of intracellular pH. *Biophys. J.* **43:**261–267
47. Todaro, G.J., Green, H. 1963. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J. Cell Biol.* **17:**299–313
48. Tupper, J.T., Zorgniotti, F., Mills, B. 1977. Potassium transport and content during G_1 and S phase following serum stimulation of 3T3 cells. *J. Cell. Physiol.* **91:**429–440
49. Ugurbil, K., Guernsey, D.L., Brown, T.R., Glynn, P., Tobkes, N., Edelman, I.S. 1981. ^{31}P NMR studies of intact anchorage-dependent mouse embryo fibroblasts. *Proc. Natl. Acad. Sci. USA* **78:**4843–4847
50. Villereal, M.L. 1981. Sodium fluxes in human fibroblasts: Kinetics of serum-dependent and serum-independent pathways. *J. Cell. Physiol.* **108:**251–259
51. Villereal, M.L., Cook, J.S. 1978. Regulation of active amino acid transport by growth-related changes in membrane potential in a human fibroblast. *J. Biol. Chem.* **253:**8257–8262

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