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Role of the Na^+/H^+ Antiport in the Regulation of the Internal pH of Ehrlich Ascites Tumor Cells in Culture

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Summary. Ehrlich ascites tumor cells contain a Na^+ uptake system, which is activated by internal protons and is inhibited by amiloride with an IC_{50} of 25 μM and by dimethylamiloride with an IC_{50} of 0.6 μM at 1 mM external Na^+. Decrease of external Na^+ or addition of amiloride is followed by a decrease of internal pH. Taken together, these findings suggest the presence of an operative Na^+/H^+ antiport system, which is involved in the regulation of internal pH. We cannot find a significant contribution of a proton pump activated by glycolysis to the pH gradient. At an external pH between 7.0 and 7.6, quiescent cells are more alkaline than exponentially growing cells (0.1 to 0.17 units). Accordingly, an increase of the affinity of the Na^+/H^+ antiport for internal protons in quiescent cells is demonstrated by the following findings: 1. The internal pH, at which the half-maximal activation of the amiloride-sensitive Na^+ uptake occurs, is shifted from 6.85 to 7.1 at 1 mM external Na^+. 2. The threshold value of external pH, below which a pronounced effect of amiloride on steady-state internal pH is observed, is shifted from 7.0 in growing to 7.5 in quiescent cells at physiological Na^+ concentrations. Therefore, we conclude that quiescent Ehrlich ascites tumor cells raise their internal pH by increasing the affinity of their Na^+/H^+ antiporter to internal protons. The Na^+/H^+ antiport cannot be activated further by addition of serum growth factors to quiescent cells. All experiments were performed at bicarbonate concentrations in the medium which do not exceed 0.5 mM. The data are discussed in view of existing models of mitogenic activity of transitory pH changes.

Key Words Na^+/H^+ antiport · pH regulation · glycolysis · Ehrlich ascites tumor cells · growth control · mitogen action

Materials and Methods

General

All experiments were done at 37°C. The statistical significance of results is expressed as mean ± SEM with the number of determinations in parentheses.

Cell Culture

Ehrlich ascites tumor cells were cultured in Eagle's minimal essential medium with Earle's salts in 2% CO_2/98% air at 36.5°C. The medium was buffered with 20 mM morpholinopropanesulfonic acid (pH 7.35 at 20°C) and supplemented with 7% serum. 0.5 mM $NaHCO_3$ was added as a nutrient. Serum consisted of a mixture of 5 parts calf serum and 2 parts fetal calf serum. Six mg penicillin G and 100 mg streptomycin were added per liter medium. Quiescent cells were obtained by incubating cells in a medium in which serum concentration was reduced to 0.5% for 24 hr (Live & Kaminskas, 1975). Compared to cells kept at 7% serum, these cells incorporate less than 20% of [^{3}H]thymidine into DNA. Since under these conditions there is no detectable increase in cell number during the 48 hr, the residual incorporation rate of thymidine in these cells is most likely due to unscheduled DNA synthesis.

Na^+ Uptake

Unidirectional Na^+ uptake was measured with ^{22}Na. The amount of intracellular ^{22}Na was determined with the silicon oil layer technique. 100 to 200 μl, containing 4×10^5 to 2×10^6 cells, were transferred to 400 μl centrifuge tubes containing 20 μl detergent solution (2 M NaCl, 40 mM EDTA, 0.2% sodium N-lauryl sarcosine, pH 10) and an upper phase of 100 μl silicon oil (10 parts AR 200/3 parts AR 20) and centrifuged in a Beckman Microfuge B for 20 sec at 10,000 × g. The tubes were sliced at the position of the silicon oil phase and the radioactivity of both parts was measured in a liquid scintillation counter. The water volume in the pellet was calculated from the distribution of [^{3}H] H_2O between pellet and supernatant. In a separate experiment with [^{3}H] inulin, the part of extracellular volume in the pellet was measured (15 to 21% of total water volume). Intracellular volume was taken as difference between the inulin volume and water volume in the pellet. The time of Na^+ uptake is defined by the interval between addition of ^{22}Na and the start of the centrifugation. Usually uptake of a 2-min period was measured. At 1 mM external Na^+, uptake is linear over a 4-min period (*data not shown*).

Internal pH

For measurement of internal pH the distribution of the weak acid [7-^{14}C] benzoic acid was determined. Benzoic acid was added to the suspension of cells of 0.1 μCi/ml (final concentration 5.2 μM) together with [^{3}H] H_2O for the measurement of cell volume. Equilibrium between intra- and extracellular space was established at least 30 sec after addition. The intracellular amount of ^{3}H and ^{14}C was determined with the silicon oil layer technique as

Table 1. Intracellular pH derived with the benzoic acid probe after treatment with nigericin at 135 mM external K^+[a]

External pH	Internal pH
6.50	6.54
7.00	7.01
7.50	7.44
8.00	7.95

[a] Exponentially growing cells from culture were washed two times with a solution containing 10 μg/ml nigericin, 135 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$ and either 20 mM piperazine-N,N' *bis* (2-ethanesulfonic acid) (PIPES) (pH 6.5 and 7.0) or 20 mM HEPES (pH 7.5 and 8.0) and incubated in this solution for 20 min. Each value represents the mean of two determinations.

described above. Both parts of the tube were transferred into separate scintillation vials, 0.5 ml 1 N $HClO_4$ and 4 ml of a toluene-based Triton X-100 scintillation cocktail were added and radioactivity was assayed by liquid scintillation counting. The internal pH was calculated with the equation

$$pH_i = 4.2 + \log_{10}[(10^{pH_e-4.2} + 1)B_i/B_e - 1] \quad (1)$$

where the indices i and e mean internal and external, respectively, and B (in cpm/μl) the concentration of benzoic acid. 4.2 is the pK_a value for benzoic acid (Allemain, Paris & Pouysségur, 1984). The equation is a rearranged form of an expression given by Roos and Boron (1981).

Table 1 shows that the addition of the K^+/H^+ ionophore nigericin to Ehrlich ascites tumor cells at 135 mM external K^+ is abolishing the pH gradient. Since under these conditions the proton distribution ratio should equal the K^+ distribution ratio (Thomas et al., 1979) this finding is expected and confirms the validity of the measured internal pH values. Schuldiner and Rozengurt (1982) and Frelin et al. (1985) have compared the values of internal pH obtained with benzoic acid and 5,5-dimethyloxazolidine-2,4-dione and have found no significant differences.

Determination of Intracellular Na^+

Intracellular Na^+ was measured with atomic absorption spectroscopy. Cells were separated from medium with the silicon oil layer technique as described above with the modification that cells were centrifuged into 20 μl 0.7 M $HClO_4$ instead of the detergent solution. Intracellular volume was determined with an identical aliquot of cells as described above.

Materials

[^{3}H] H_2O, [7-^{14}C] benzoic acid and ^{22}Na were obtained from New England Nuclear, Dreieich, FRG. [^{3}H] inulin and [methyl ^{3}H] thymidine were from the Radiochemical Centre, Amersham, England, silicon oils AR 20 and AR 200 from Wacker Chemie, Vienna, Austria. Amiloride and dimethylamiloride were a gift from Merck, Sharp & Dohme, Vienna.

Introduction

Numerous authors have demonstrated that various eucaryotic cells can efficiently regulate their internal pH in response to intracellular acid or alkaline loads (Roos & Boron, 1981; Bowen & Levinson, 1984; Moolenaar et al., 1984*a*; Simchowitz & Roos, 1985). The sensitivity of many cellular processes to a variation of pH requires a tight regulation of intracellular pH. Increasing evidence is accumulating that cells cannot only maintain their steady-state pH value but also are able to change their internal pH and adapt new steady-state levels in response to external triggers like fertilization (Johnson et al., 1976), or growth factors (Schuldiner & Rozengurt, 1982; Allemain, Paris & Pouysségur, 1984; Moolenaar et al., 1984*b*). In all cases a cytoplasmic alkalinization together with a stimulation of the Na^+/H^+ antiport is observed. It has been proposed (Winkler & Grainger, 1978; Pouysségur et al., 1984) that this alkalinization is a necessary event in the induction of the cellular responses following fertilization or mitogenic stimulation. Paris and Pouysségur (1984) have demonstrated that in Chinese hamster lung fibroblasts the activation of the Na^+/H^+ antiporter by

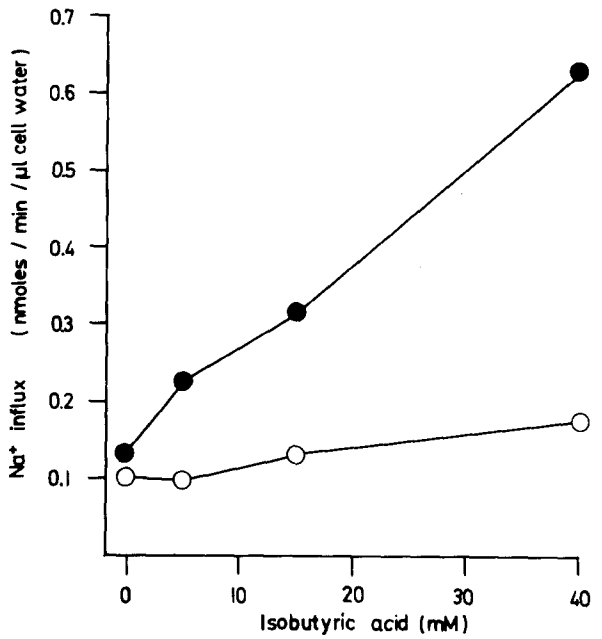

Fig. 1. Effect of isobutyric acid on sodium uptake at 1 mM external Na^+. 1 to 2 × 10^6 exponentially growing cells from culture were centrifuged for 5 min at 200 × g and resuspended in 1 ml buffer containing (in mM): 140 choline chloride, 5.4 KCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 5.0 glucose and 25 HEPES/Tris, pH 7.4. After 1 min cells were centrifuged (70 × g, 90 sec) and resuspended in 250 μl of the same buffer containing the indicated concentrations of choline isobutyric acid instead of equal concentrations of choline chloride with (○) or without (●) 0.6 mM amiloride. After 1 min an aliquot (165 μl) was added to 55 μl solution containing ^{22}Na and NaCl (final concentrations of mixture 2 μCi/ml and 1 mM). After 2 min Na^+ influx was determined with the silicon oil layer technique as described in Materials and Methods. Results are expressed as means of at least four determinations

growth factors is due to an increase of its affinity for intracellular H^+.

Transformed cell lines can escape the control of external growth factors through autonomous self-stimulation by autocrine production of growth factors or modifications of the sequence of events after binding of growth factors to their receptors (Sporn & Roberts, 1985). Since the effect of growth factors on the regulation of internal pH seems to be closely correlated to their mitogenic effect, it is an intriguing question whether the altered dependence of tumor cells on external growth factors is reflected by a change of their pH regulation properties. Therefore we have studied the regulation of internal pH in Ehrlich ascites tumor cells (EATC). We have focused our interest on the Na^+/H^+ antiport and its contribution to the inward-directed proton gradient in these cells.

In this study we present evidence that in Ehrlich ascites tumor cells in culture at bicarbonate concentrations $\leqq 0.5$ mM the internal pH is regulated, depending upon growth conditions, mainly by the Na^+/H^+ antiport system. However, in contrast to fibroblasts (Paris & Pouysségur, 1984), quiescent Ehrlich cells do not activate their Na^+/H^+ antiport upon serum addition. Unexpectedly, the internal pH of quiescent Ehrlich cells was found to be more alkaline than the pH of exponentially growing cells.

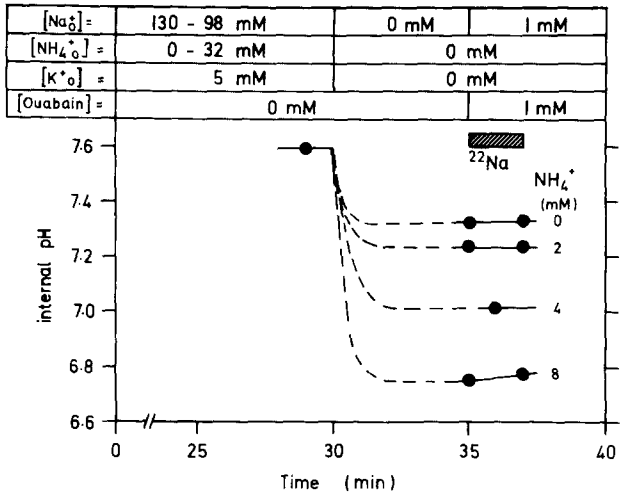

Fig. 2. Variation of internal pH at constant external pH with the ammonium chloride preload technique. Cells from culture were centrifuged for 5 min at 200 × *g* and resuspended in buffer 1 (130 to 98 mM NaCl, 0 to 32 mM NH_4Cl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 20 mM HEPES/Tris, pH 7.4), centrifuged for 90 sec at 70 × *g* and resuspended in the same buffer. After 30 min, buffer 1 was replaced by two cycles of centrifugation at 70 × *g* for 90 sec with buffer 2 (135 mM choline chloride, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 20 mM HEPES/Tris, pH 7.4). One min after the second resuspension ^{22}Na was added together with NaCl and ouabain to a final concentration of the mixture of 1 mM each. The Na^+ uptake of a 2-min interval (indicated by the hatched box in the figure) was measured with the silicon oil technique. At the indicated times the internal pH (●) after preloading with different concentration of ammonium chloride was determined with the benzoic acid probe. Preloading cells with 16 and 32 mM ammonium chloride leads to similar pH changes as with 8 mM (*not shown*). Both methods are described in Materials and Methods. At the top of the diagram the variation of composition of the main components of the external medium during the experiment is illustrated

Results

Ehrlich Ascites Tumor Cells have an Amiloride-Sensitive Na^+ Uptake which is Activated by Internal Protons

At 130 mM external Na^+ we cannot detect a Na^+ influx which is sensitive to 1 mM amiloride or 0.5 mM dimethylamiloride in Ehrlich ascites tumor cells (*data not shown*). This observation is similar to the situation in Chinese hamster lung fibroblasts (Paris & Pouysségur, 1984). At 1 mM external Na^+, however, an amiloride-inhibitable sodium influx is measurable. We can demonstrate that this transport is activated by an increase of internal proton concentration in two different types of experiment:

One way of increasing the internal proton concentration at constant external pH is by treating cells with different concentrations of isobutyric acid (Moolenaar et al., 1984*a*). The weak acid penetrates the cell membrane predominantly in the nondissociated form and leads to an intracellular acidification after dissociation in the cytosol. Figure 1 shows that the amiloride-sensitive Na^+ transport is stimulated with increasing concentration of isobutyric acid at a constant external pH of 7.4.

Another way of obtaining intracellular acidification at constant external pH is to preload cells with ammonium by incubation with varying concentrations of ammonium chloride (Boron, 1977). After removal of the external ammonium chloride the uncharged NH_3 molecules permeate the plasma membrane in the direction of the concentration gradient and leave protons behind. Figure 2 illustrates the experimental procedure which represents a modified version of the technique described by Paris and Pouysségur (1984). The decrease of internal pH is proportional to the amount of ammonium chloride in the preincubation medium. At 1 mM Na^+ the recovery of internal pH after removal of ammonium chloride is slow, which makes it possible to determine the sodium uptake rate at a defined internal pH. ^{22}Na is added to a K^+-free and 1 mM ouabain-containing medium in order to inhibit the Na^+/K^+-ATPase. The results (Fig. 3) support the data shown in Fig. 1 by demonstrating the presence of an amiloride-sensitive Na^+ transport system, which is activated by internal protons.

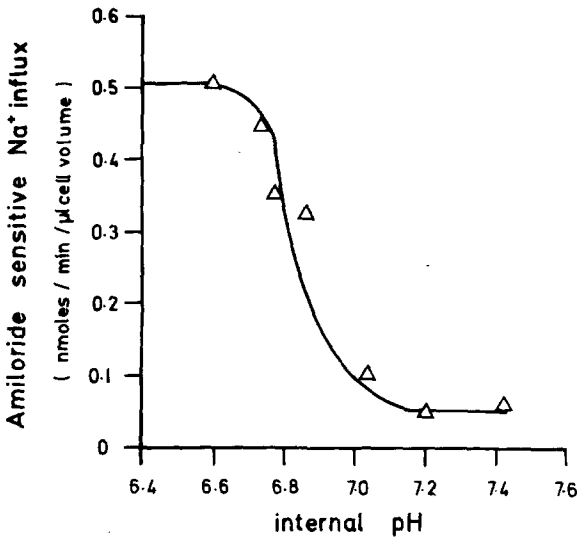

Fig. 3. Stimulation of amiloride-sensitive sodium influx by internal protons. Exponentially growing cells were loaded with protons as described in Fig. 2. The intracellular pH and sodium uptake were determined in parallel samples with the silicon oil layer technique as described in Materials and Methods. The radioactivity was added at the same time to each sample. Duplicate 200-μl samples were layered on top of centrifuge tubes prefilled with detergent and silicon oil as described in Materials and Methods. One min after addition of radioactivity, samples with ^{14}C benzoic acid and ^3H H_2O were centrifuged, while samples which received ^{22}Na were centrifuged after 2 min. Intracellular volume, intracellular pH and the Na^+ uptake per minute and μl cell volume were calculated as described in Materials and Methods. The amiloride-resistant Na^+ influx at 1 mM amiloride was 0.045 ± 0.015 (4) nmol Na^+/(μl cell volume · min) and was not affected by a variation of internal pH or the presence of serum. This value was subtracted from the total Na^+ influx to obtain the amiloride-sensitive Na^+ influx

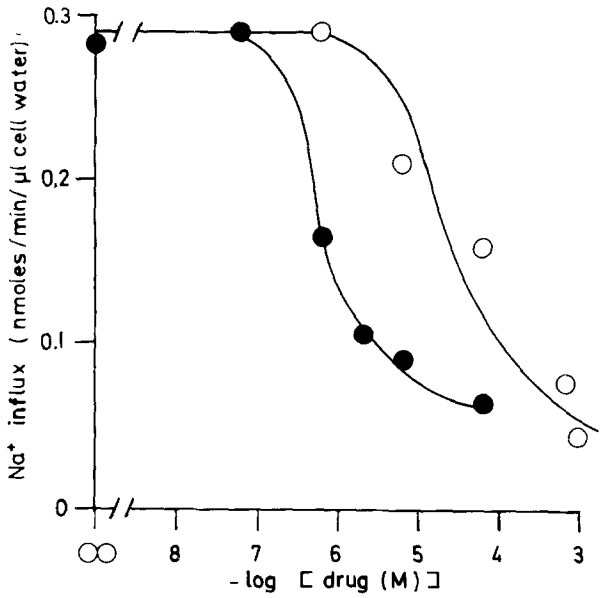

Fig. 4. Inhibition of Na^+ influx by amiloride (○–○) and dimethylamiloride (●–●) in EATC pretreated with 16 mM NH_4Cl. The experimental conditions are the same as in Fig. 2. Exponentially growing cells were preloaded with 16 mM NH_4Cl and the drugs were added 5 min before radioactivity at the concentrations indicated in the diagram. Sodium influx was determined with the silicon oil layer technique as described in Materials and Methods. IC_{50} values for the inhibition of Na^+ influx were determined from this figure by taking 100% inhibition as the one found in the presence of 1 mM amiloride (0.045 ± 0.015 (4) nmol Na^+/(μl cell volume · min))

Pharmacological Evidence that the Na^+ Transport System which is Activated by Internal Protons is a Na^+/H^+ Antiport System in Ehrlich Ascites Tumor Cells

Figure 4 shows that the Na^+ transport system of Ehrlich ascites tumor cells shown above can be inhibited at 1 mM external Na^+ by amiloride and dimethylamiloride with IC_{50} values of 25 and 0.6 μM, respectively. A similar difference in the potency of the two drugs has been demonstrated in other cell types (Allemain, Franchi, Cragoe & Pouysségur, 1984; Vigne, Frelin, Cragoe & Lazdunski, 1984). The authors show that amiloride and its dimethyl-derivative specifically inhibit the Na^+/H^+ antiporter. The similar pharmacological properties suggest a close relationship of the system in Ehrlich ascites tumor cells to those described by Allemain et al. (1984) and Vigne et al. (1984).

The Magnitude of the pH Gradient is Dependent on External Na^+

Figure 5 shows that at 130 mM external Na^+ concentration EATC maintain an outward pH gradient of 0.21. A decrease of external sodium is followed by a decrease of the pH gradient. This finding is in accordance with the assumption that these cells use, at least in part, their inward-directed Na^+ gradient to maintain their inward-directed proton gradient. The magnitude of the sodium gradient would be high enough for the generation of the observed pH gradient: 20 min after resuspension in the buffer described in the legend of Fig. 5 (130 mM external Na^+) we measure an internal Na^+ of 22 ± 2 mM (8). This means that the sodium gradient would be sufficient to drive the internal pH to 8.17 when the external pH is 7.4.

Lactic Acid Production and Regulation of Internal pH

As shown in Fig. 6, the difference in the rates of proton and lactate extrusion is not significant. The

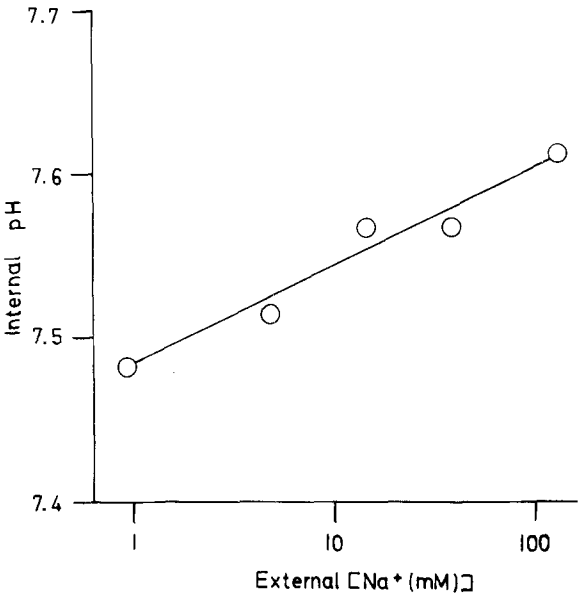

Fig. 5. Dependence of internal pH on external Na^+ concentration. Exponentially growing cells were washed two times with a buffer containing NaCl of the indicated concentrations, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 20 mM HEPES/Tris, pH 7.4 and choline chloride to give 130 mM NaCl plus choline chloride. The interal pH was determined 20 min after resuspension in this buffer with the benzoic acid probe as described in Materials and Methods. Results are expressed as means of two determinations

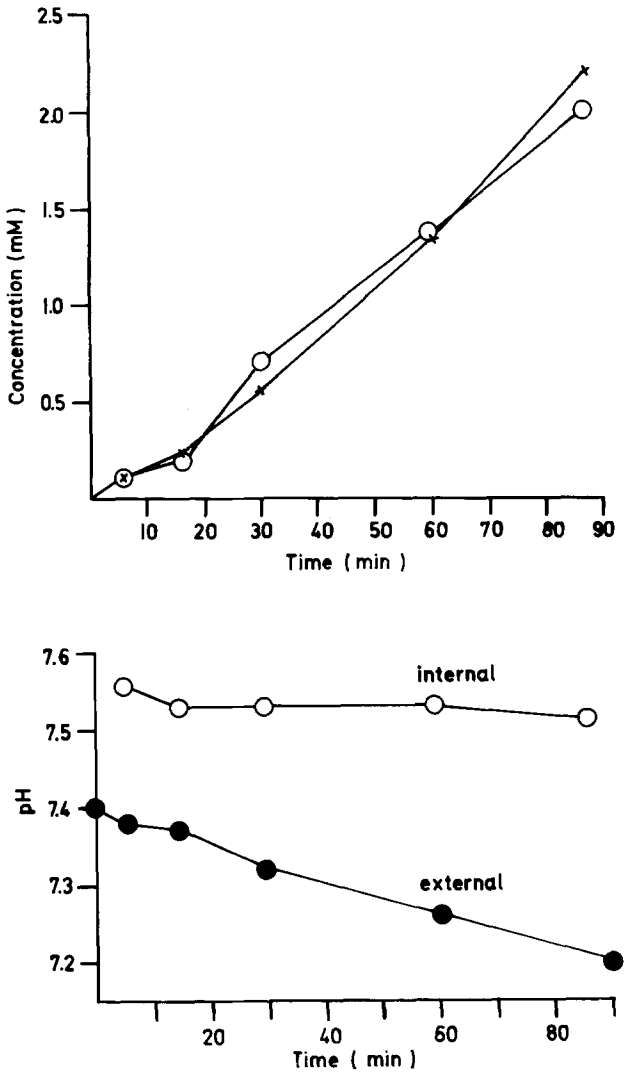

Fig. 6. Rate of lactate and proton production of exponentially growing cells. Exponentially growing cells were washed with Eagle's minimal essential medium buffered with MOPS, pH 7.4, and resuspended at a cell density of 1.86×10^6 cells/ml in the same medium. Mean cell volume, determined with $^3H\ H_2O$ and ^3H inulin as described in Materials and Methods under Na^+ uptake, was 1.99 ± 0.06 (12) $\mu l/10^6$ cells. At the indicated times after resuspension 1-ml aliquots were centrifuged at 10,000 × *g* for 15 sec and the supernatant used for determination of external pH (●); lower panel) and lactate concentration in the medium (×; upper panel). Another aliquot was taken for determination of internal pH (○; lower panel) with benzoic acid as described in Materials and Methods. The amount of protons released into the medium (○; upper panel) was determined by measuring the amount of 1 N HCl needed to decrease the pH to the same extent. Lactate was measured as described by Gutmann and Wahlefeld (1974)

rates in Eagle's minimal essential medium are 5.44 ± 0.29 (10) mmol/(min · liter cell water). Therefore, we conclude that the production of lactic acid represents the main contribution to the release of protons into the medium. There are different views in the literature describing how lactate is extruded by EATC. Spencer & Lehninger (1976) demonstrated the existence of an electroneutral carrier operating in a 1:1 symport of lactate with protons or 1:1 antiport of lactate with OH^- ions. Although not suggested by the authors, a carrier which is transporting the free acid would be consistent with the experiments described in this work. Heinz, Sachs and Schafer (1981) have presented evidence for an active electrogenic proton pump in EATC which is activated by glycolysis. They argue that the hyperpolarizing action of that pump would stimulate the outward transport of lactate by increasing the outward electrochemical gradient. If one considers that a pump with the properties described by Heinz et al. (1981) would have to transport 5.44 mmol protons/(min · liter cell water) under steady-state conditions it should contribute significantly to the pH gradient of EATC. As this pump is described to be operative during glycolysis only, we measured the pH gradient of cells with no lactate production. As depicted in Fig. 7 these cells maintain a pH gradient which is similar to the one in Eagle's minimal essential medium (Fig. 6). A stimulation of glycolysis causes

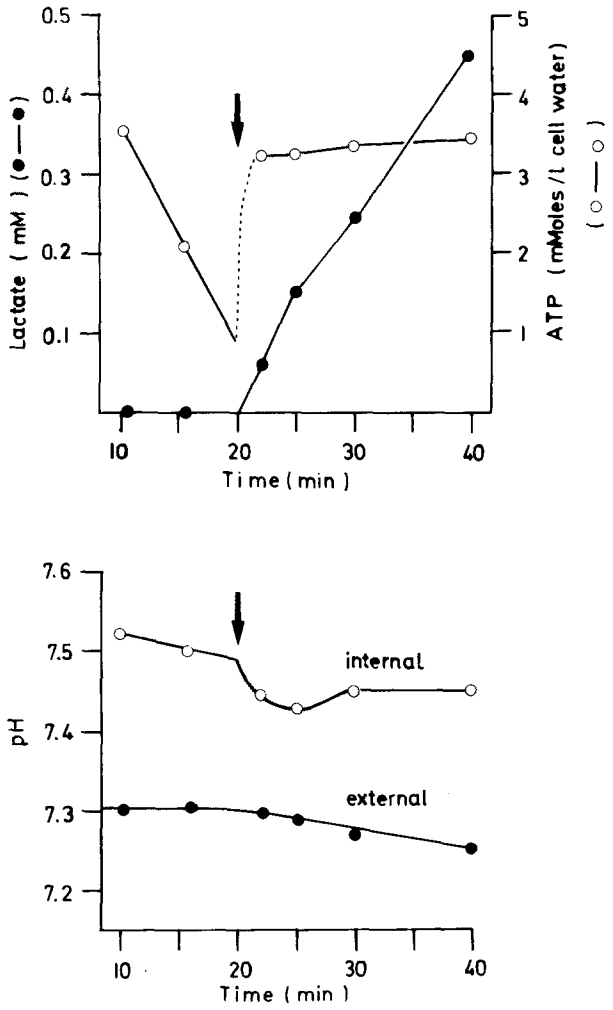

Fig. 7. Dependence of pH gradient on glycolysis. Lactate concentration in the medium (●; upper panel) and external pH (●; lower panel) were measured as described in the legend of Fig. 6. For measurement of ATP (○; upper panel) 0.5-ml aliquots of the cell suspension were mixed with 0.5 ml 0.8 M $HClO_4$ at 0°C. 15 min later, after centrifugation (1 min at 10,000 × *g*) the supernatant was neutralized with 10 N KOH and taken for determination of ATP by the method of Lamprecht and Trautschold (1974). Exponentially growing cells were washed two times with a solution containing (in mM): 5 NaH_2PO_4, 1 $MgSO_4$, 130 NaCl, 5 KCl and 20 HEPES/Tris, pH 7.3. At time 0 cells were resuspended in the same buffer at a density of 4.8 × 10^6 cells per ml. Mean cellular volume was 1.51 ± 0.04 (10) µl per 10^6 cells. At the time indicated by the arrow 5 mM glucose were added. One experiment out of three similar is shown in the figure

a transitory decrease of intracellular pH; after 10 min the previous gradient is restored.

As shown in Fig. 6, a variation of the external lactate concentration between 0 and 2 mM has no significant effect on internal pH. Therefore, we conclude: a) The extrusion of lactic acid is accomplished by a transport mechanism for the undissociated acid along a steep outward-directed concentration gradient[1]. b) The regulation of the internal pH is neither affected by the rate of glycolysis (Fig. 7) nor by lactate concentration (Fig. 6). Actively glycolyzing cells employ lactic acid excretion as an additional mechanism to eliminate excess protons generated by glycolysis. However, this system cannot account for the inward-directed proton gradient.

Table 2. Benzoic acid-derived internal pH values of quiescent and exponentially growing EATC[a]

External pH	Internal pH		
	Quiescent cells	Exponential cells	pH difference
7.0	7.39 ± 0.03 (4)	7.29 ± 0.05 (4)	0.10 ($P < 0.05$)
7.3	7.68 ± 0.04 (4)	7.54 ± 0.03 (4)	0.14 ($P < 0.01$)
7.6	7.80 ± 0.02 (4)	7.63 ± 0.02 (4)	0.17 ($P < 0.01$)

[a] Exponentially growing cells or quiescent cells kept for 24 hr in 0.5% serum were centrifuged (200 × *g*, 5 min) and suspended in Eagle's minimal essential medium with Earle's salts buffered with 20 mM morpholinopropanesulfonic acid (MOPS; pH 7.0 and 7.3) or 20 mM N-(2-hydroxyethyl)-piperazine-N'-3-propanesulfonic acid (EPPS; pH 7.6). Serum concentration was 0.5% (quiescent cells) and 7% (exponentially growing cells). After 30 min the internal pH was measured with the benzoic acid method as described in Materials and Methods. Results are expressed as means ± SEM with the number of determinations in parentheses.

In EATC the Regulation of Internal pH is Different in Quiescent and Growing Cells

Table 2 shows that quiescent cells are between 0.1 and 0.17 pH units more alkaline at an external pH between 7.0 and 7.6 than the exponentially growing cells. The dependence of the difference between internal and external pH on external proton concentration, covering a more extended pH range, is depicted in Fig. 8 for growing cells (panel *A*) and for resting cells (panel *B*).

The effect of amiloride on the steady-state internal pH for exponentially growing cells is shown in Fig. 8 (panel *A*). At external pH values above 7.0, amiloride decreases internal pH by 0.1 units. Between an external pH of 6.0 to 7.0 the drug has a pronounced effect on internal pH, while a further increase in external protons is diminishing again the effect of the drug on internal pH. We interpret these findings in the following manner: At pH values

[1] Thomas, Buchsbaum, Zimniak and Racker (1979) have measured an intracellular concentration of lactate in EATC of 100 mM.

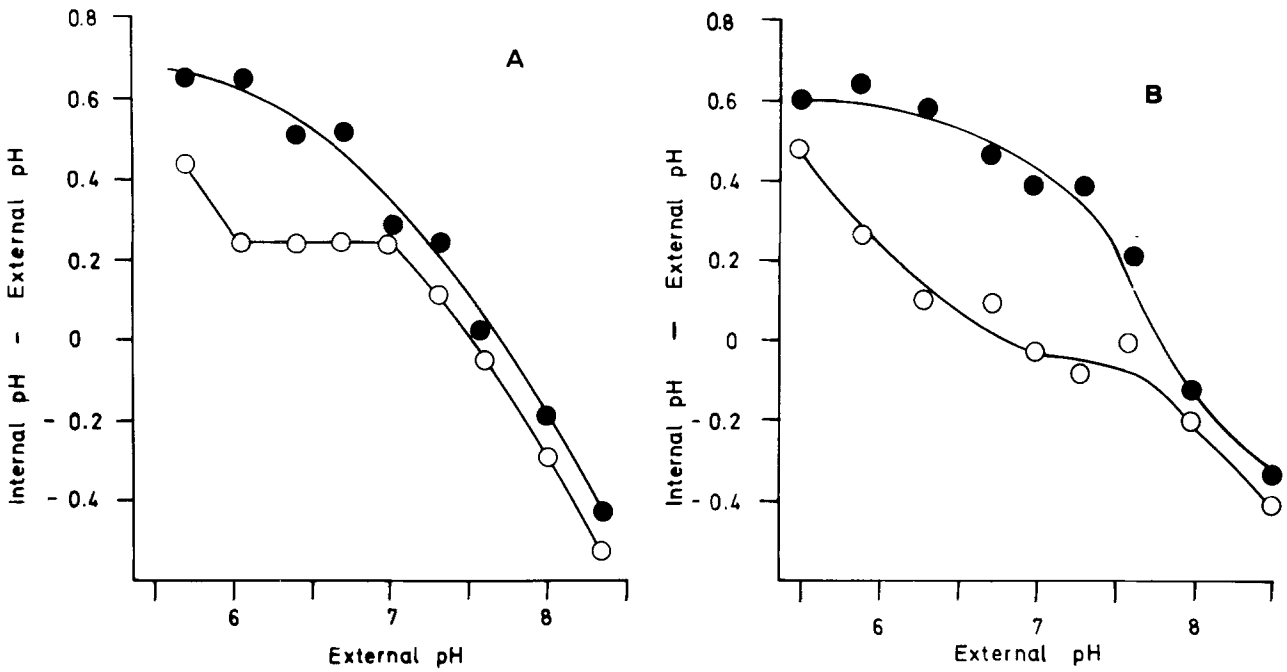

Fig. 8. Dependence of internal pH on external pH. (*A*) Exponentially growing cells were washed once with Eagle's minimal essential medium with Earle's salts, 7% serum and 20 mM of the following buffers: 2(N-morpholino)ethanesulfonic acid (MES), pH 5.5 to 6.1; piperazine-N,N' *bis* (2-ethanesulfonic acid) (PIPES), pH 6.3 to 6.7; morpholinopropane-sulfonic acid (MOPS), pH 7.0 to 7.3; N-(2-hydroxyethyl)-piperazine-N'-3-propanesulfonic acid (EPPS), pH 7.6 to 8.5. (*B*) Quiescent cells, which have been kept in culture for 24 hr in a medium with 0.5% serum were subjected to the same experimental procedure, the difference being that the medium contained only 0.5% serum. The cells were resuspended in the same medium with or without 1 mM amiloride. After 30 min the internal pH was determined with benzoic acid as described in Materials and Methods. (○–○): + 1 mM amiloride; (●–●) no amiloride

above 7.0 the Na^+/H^+ antiport is operating at low speed. Decreasing external pH below 7.0 is stimulating the system due to the increased proton influx under these conditions (Bowen & Levinson, 1984). Therefore, a blockade of the system by amiloride has a pronounced effect on the pH gradient. At external pH values below 6.0 the system is already operating at maximal speed, and can, therefore not compensate a further increase of proton influx.

There is a different situation is quiescent cells (Fig. 8, panel *B*): Amiloride has a pronounced effect on the internal pH already at external pH values below a threshold value of 7.5, indicating that in these cells the Na^+/H^+ antiporter is activated at much lower proton concentrations in the medium.

Increased Affinity of the Na^+/H^+-Antiport System for Internal Protons is Responsible for the Differences of Internal pH in Quiescent and Growing Cells

Figure 9 demonstrates a difference in the dependence of amiloride-sensitive Na^+ influx on internal protons between quiescent and exponentially growing Ehrlich cells. The internal pH, at which half-maximal stimulation of the amiloride-sensitive Na^+ influx is seen, is shifted to more alkaline values in quiescent cells. In exponentially growing cells this internal pH was determined as 6.85, whereas in quiescent cells a shift of 0.25 units to 7.1 is to be seen.

An alternative explanation for the increased activity of the Na^+/H^+ antiporter in quiescent cells would be a decrease in the intracellular Na^+ concentrations in quiescent cells. This, however, is not the case. The measured intracellular Na^+ concentrations under cell culture conditions were determined as 0.049 ± 0.03 (6) moles/liter cell water for exponentially growing cells and 0.061 ± 0.08 (4) moles/liter cell water for quiescent cells, respectively. The higher Na^+ concentration in quiescent cells corresponds to the increased Na^+ influx by the activated Na^+/H^+ antiporter. The combined results from Figs. 8 and 9 strongly suggest that the differences in regulation of internal pH are a consequence of a change of the sensitivity of the antiport system to stimulation by internal protons.

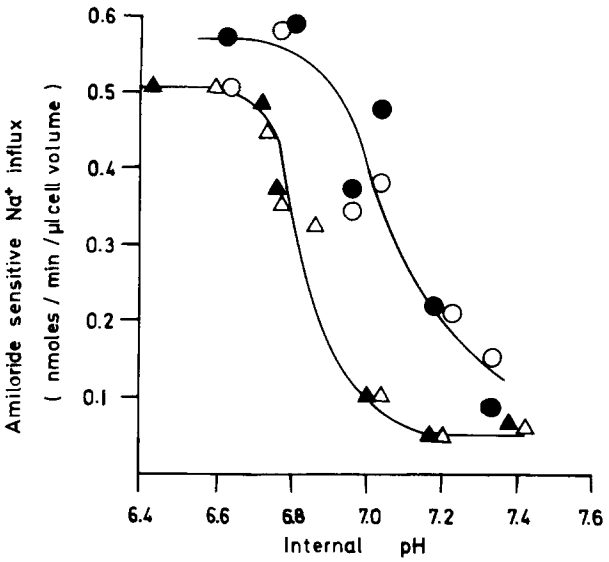

Fig. 9. Effect of serum and growth conditions on the stimulation of the amiloride-sensitive sodium influx by internal protons. Amiloride-sensitive Na^+ influx and internal pH at 1 mM external pH were measured simultaneously 35 min after removal of cells from culture as described in Figs. 2 and 3. Exponentially growing cells: triangles; quiescent cells: circles; filled symbols: addition of 10% serum, dialyzed against 135 mM choline chloride, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 20 mM HEPES/Tris, pH 7.4, 5 min before radioactivity; open symbols: no addition of serum. The dialyzed serum is able to stimulate cell proliferation of quiescent cells (*data not shown*)

The Change in Dependence on Internal Protons of the Antiport Cannot be Explained by a Short-Term Effect of Serum Constituents

Figure 9 also shows that an addition of serum 5 min before addition of radioactivity has no significant effect on the dependence of the antiport on internal protons. This is true for quiescent and growing cells. The finding raises questions regarding the nature of regulatory mechanism underlying the observed phenomenon. This will be outlined in the Discussion.

Discussion

In this study we present evidence for the operation of a Na^+/H^+ antiport system in Ehrlich ascites tumor cells. Demonstration of this system is based on isotopic flux studies with ^{22}Na, on pharmacological properties and on measurements of internal pH.

Our results indicate a major contribution of the Na^+/H^+ antiport to the maintenance and regulation of the inward-directed proton gradient in Ehrlich ascites cells. The regulation of the internal pH depends on external Na^+, which is in accordance with data obtained with fibroblasts (Moolenaar et al., 1984*a*), neutrophils (Simchowitz & Roos, 1985) and chick skeletal muscle cells (Vigne, Frelin & Lazdunski, 1984) for which an operative Na^+/H^+-antiport system has been demonstrated. A blockade of the system with amiloride affects the steady-state internal pH. In fibroblasts (Moolenaar et al., 1984*a*) and neutrophils (Simchowitz & Roos, 1985) such an effect is seen only after loading the cells with acid. The effect of amiloride on internal pH on EATC under physiological conditions is not due to a higher affinity of amiloride to the antiport system of Ehrlich ascites tumor cells, since the IC_{50} for the Ehrlich system is 25 µM compared to 5 µM in fibroblasts (Vigne Frelin & Lazdunski, 1984), but seems to reflect a higher activity of this system under the conditions used.

Bowen and Levinson (1984) have shown that in EATC a decrease of external pH leads to an increase in proton influx. As depicted in Fig. 8, a decrease of external pH amplifies the amiloride-induced decrease of internal pH. These findings strongly suggest that it is mainly the Na^+/H^+ antiport which is stimulated under these conditions.

A Na^+-dependent HCO_3^-/Cl^- antiport system, which is sensitive to stilbene derivatives has been described as another candidate for the regulation of internal pH (for a review *see* Roos & Boron, 1981). Hoffmann (1982) has demonstrated the existence of a stilbene derivative sensitive anion exchange system in Ehrlich cells. Since our cells are growing in culture also at atmospheric CO_2 and in the absence of HCO_3^- (Doppler, *unpublished*), we conclude that this system is not essential for the pH regulation under the culture conditions used. Accordingly, Allemain et al. (1985) have reported that a Na^+-dependant anion exchange system is involved in pH regulation of a Na^+/H^+ antiport-deficient mutant of fibroblasts, but is not essential for the pH regulation of the parental strain.

As shown in Figs. 6 and 7 neither external lactate concentration nor lactate release rate have a significant effect on internal pH. It is concluded therefore, that an electrogenic proton pump, which is activated by glycolysis and described by Heinz et al. (1981), does not significantly contribute to the pH gradient.

A major finding of this study is the dependence of internal pH on growth conditions. We can demonstrate that Ehrlich ascites tumor cells can regulate their internal pH by changing the affinity of their Na^+/H^+ antiport for internal protons. Paris and Pouysségur (1984) have reported that the growth factor-induced alkalinization in fibroblasts is based on a similar change of affinity for internal protons. Our data implicate that this might be a common mechanism for the regulation of the steady-state value of internal pH. However, the

conditions at which the change in affinity occurs are completely different in fibroblast and Ehrlich ascites tumor cells. While an increase in affinity for internal protons is induced in less than one minute after addition of growth factors to quiescent fibroblasts (Paris & Pouysségur, 1984), Ehrlich cells do not respond to the addition of serum growth factors after 5 min. In contrast to fibroblasts, in Ehrlich cells an increase in the affinity for internal protons is observed in quiescent cells compared to exponentially growing cells. This finding is unexpected, since in the systems studied so far quiescent cells exhibit lower internal pH values and raise their internal pH after growth stimulation.

Autonomous mechanisms of activation of the Na^+/H^+ antiport provide a possible explanation for the increased internal pH in quiescent Ehrlich cells. Although Ehrlich cells are dependant on serum for maintenance of cell growth, the growth factor dependence may be different compared to fibroblast cell lines. The following situation may exist in Ehrlich ascites tumor cells: Quiescent cells can activate their antiport system by autonomous mechanisms, but need (an) additional serum growth factor (s) for initiation of DNA-synthesis. The internal pH is higher than in exponentially growing cells as the absence of the additional growth factor(s) leads to an arrest of cells in a phase characterized by a high activity of the Na^+/H^+ antiporter. An exponentially growing population would contain cells in all cell cycle phases, thus the percentage of cells present in the state with the activated Na^+/H^+ antiporter should be lower. The validity of this assumption is the subject of current investigations.

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References

Allemain, G. L', Franchi, A., Cragoe, E., Jr., Pouysségur, J. 1984. Blockade of the Na^+/H^+ antiport abolishes growth factor-induced DNA synthesis in fibroblasts. *J. Biol. Chem.* **259:**4313–4319

Allemain, G. L', Paris, S., Pouysségur, J. 1984. Growth factor action and intracellular pH regulation in fibroblasts. *J. Biol. Chem.* **259:**5809–5815

Allemain, G. L', Paris, S., Pouysségur, J. 1985. Role of a Na^+-dependent Cl^-/HCO_3^- exchange in regulation of intracellular pH in fibroblasts. *J. Biol. Chem.* **260:**4877–4883

Boron, W.F. 1977. Intracellular pH transients in giant barnacle muscle fibres. *Am. J. Physio.* **233:**C61–C73

Bowen, J.W., Levinson, C. 1984. H^+ transport and the regulation of intracellular pH in Ehrlich ascites tumor cells. *J. Membrane Biol.* **79:**7–18

Frelin, C., Vigne, P., Lazdunski, M. 1985. The role of the Na^+/H^+ exchange system in the regulation of the internal pH in cultured cardiac cells. *Eur. J. Biochem.* **149:**1–4

Gutmann, I., Wahlefeld, A.W. 1974. L(+)-Lactat: Bestimmung mit Lactat-Dehydrogenase und NAD. *In:* Methoden der enzymatischen Analyse. H.U. Bergmeyer, editor. pp. 1510–1514. Verlag Chemie Weinheim/Bergstr.

Heinz, A., Sachs, G., Schafer, J.A. 1981. Evidence for activation of an active electrogenic proton pump in Ehrlich ascites tumor cells during glycolysis. *J. Membrane Biol.* **61:**143–153

Hoffmann, E.K. 1982. Anion exchange and anion-cation cotransport systems in mammalian cells. *Philos. Trans. R. Soc. London B* **299:**519–535

Johnson, J.J. Epel, D., Paul, M. 1976. Intracellular pH and activation of sea urchin eggs after fertilization. *Nature (London)* **262:**661–664

Lamprecht, W., Trautschold, I. 1974. Bestimmung mit Hexokinase und Glucose-6-phosphat-Dehydrogenase. *In:* Methoden der enzymatischen Analyse. H.U. Bergmeyer, editor. pp. 2151–2160. Verlag Chemie Weinheim/Bergstr.

Live, T.R., Kaminskas, E. 1975. Changes in adenylate energy charge in Ehrlich ascites tumor cells deprived of serum, glucose, or amino acids. *J. Biol. Chem.* **250:**1786–1789

Moolenaar, W.H., Tertoolen, L.G.J., de Laat, S.W. 1984*a*. The regulation of cytoplasmic pH in human fibroblasts. *J. Biol. Chem.* **259:**7563–7569

Moolenaar, W.H., Tertoolen, L.G.J., de Laat, S.W. 1984*b*. Phorbol ester and diacylglycerol mimic growth factors in raising cytoplasmic pH. *Nature (London)* **312:**371–374

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

Pouysségur, J., Sardet, C., Franchi, A., Allemain, G. L', 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

Roos, A., Boron, W.F. 1981. Intracellular pH. *Physiol. Rev.* **61:**296–434

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.

Simchowitz, L., Roos, A. 1985. Regulation of intracellular pH in human neutrophils. *J. Gen. Physiol.* **85:**443–470

Spencer, T., Lehninger, A.L. 1976. L-lactate transport in Ehrlich ascites tumor cells. *Biochem. J.* **154:**405–414

Sporn, M.B., Roberts, A.B. 1985. Autocrine growth factors and cancer. *Nature (London)* **313:**745–747

Thomas, J.A., Buchsbaum, R.N., Zimniak, A., Racker, E. 1979. Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. *Biochemistry* **18:**2210–2218

Vigne, P., Frelin, C., Cragoe, E.J., Jr., Lazdunski, M. 1984. Structure-activity relationships of amiloride and certain of its analogues in relation to the blockade of the Na^+/H^+ exchange system. *Mol. Pharmacol.* **25:**131–136

Vigne, P., Frelin, C., Lazdunski, M. 1984. The Na^+-dependent regulation of the internal pH in chick skeletal muscle cells. The role of the Na^+/H^+ exchange system and its dependence of internal pH. *EMBO J.* **3:**1865–1870

Winkler, M.M., Grainger, J.L. 1978. Mechanism of action of NH_4Cl and other weak bases in the activation of sea urchin eggs. *Nature (London)* **273:**536–538

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