Cytoplasmic pH, a key determinant of growth factor-induced DNA synthesis in quiescent fibroblasts

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Received 10 August 1985

In response to growth factors, quiescent fibroblast mutants lacking Na^+/H^+ exchange activity fail to elevate their cytoplasmic pH (pH_i) and to reinitiate DNA synthesis at neutral and acidic pH_o. A pH_i threshold of ~7.2 exists, below which growth factors cannot set in motion the G_0 to S phase transition. Restoration of the pH_i defect in mutant cells restores the wild-type phenotype. These findings, combined with the properties of another class of mutants able to grow at very low pH_o, demonstrate that pH_i, modulated by growth factor activation of the Na^+/H^+ antiporter, plays a determinant role in growth control.

Na^+/H^+ antiporter *pH_i* *Fibroblast mutant* *DNA synthesis* *Growth factor*

1. INTRODUCTION

Stimulation of Na^+ influx and rise in cytoplasmic pH (pH_i), first observed in sea urchin eggs after fertilization [1], have since been reported to be a ubiquitous response preceding metabolic activation of quiescent cells (reviews [2–4]). Growth-promoting agents, e.g., serum [5], purified growth factors: EGF [6], PDGF [6,7], α-thrombin [8,9], phorbol esters, diacylglycerol [11,12], concanavalin A [13], have all been shown to induce a rapid rise in pH_i of 0.1–0.3 pH units. It is now clearly established that this cytoplasmic alkalinization results from growth factor activation of a membrane-bound amiloride-sensitive Na^+/H^+ antiporter [5,8,14–18].

However, whether this rise in pH_i is necessary for mitogen action has remained an open question. We now present genetic evidence that pH_i controls the rate of entry of mitogen-stimulated quiescent cells into S phase. Prevention of mitogen-stimulated Na^+ influx and pH_i rise in a fibroblast mutant lacking Na^+/H^+ antiport activity [8,19] suppresses growth factor-induced DNA synthesis at neutral and acidic pH_o. Although a pH_i rise is not mitogenic per se, we show that its increase above a critical threshold value is a prerequisite for growth factor-stimulated quiescent cells to progress into the cell cycle.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Chinese hamster lung fibroblasts (CCL39 from ATCC) and its mutant derivatives (PS120 and AS7) were cultivated in Dulbecco's MEM supplemented with 5% fetal calf serum (FCS), 25 mM bicarbonate, at 37°C, in an atmosphere of 5% CO_2/95% air. The isolation and biochemical characterization of PS120 mutant cells lacking Na^+/H^+ exchange activity have been described [8,19]. AS7 mutant cells were selected from an ethylmethanesulfonate-mutagenized CCL39 cell population. Cells were plated at 5×10^6 cells per 100 mm dish and incubated in DMEM buffered with 30 mM Mes, pH_o 6.4 and supplemented with 10% dialyzed FCS and 100 μM uridine and hypoxanthine. Clones which arose after 2 weeks of culture in this 'acidic selective' medium were recloned (AS7) and passaged in HCO_3^--free medium, pH 6.4.

Cells were arrested in G_0/G_1 by incubation for

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00145793/85/$3.30

24 h in the total absence of serum [20] in a 1:1 mixture of DMEM/Ham's F12 medium (Gibco).

2.2. $^{22}Na^+$ uptake

Arrested confluent cells in 4.5 cm^2 diam. wells (Linbro) were preincubated for 30 min at 37°C in DMEM buffered at pH 7.8 with 30 mM Hepes. Then cells were washed twice with 130 mM choline Cl, pH 7.8 and $^{22}NaCl$ (1 μCi/ml), 2 mM $CaCl_2$, 1 mM $MgCl_2$, 130 mM choline Cl, 1 mM ouabain, 20 mM Hepes-Tris, pH 7.8 was added. Uptakes were terminated by washing the cell monolayer 4 times with ice-cold phosphate-buffered saline and total cell radioactivity was counted in a gamma counter. Amiloride-insensitive $^{22}Na^+$ uptake obtained in parallel wells in the presence of 100 μM 5-*N,N*-dimethylamiloride (gift from Dr E. Cragoe jr, Merck, Sharp & Dohme) were subtracted from total $^{22}Na^+$ uptake.

2.3. *Intracellular pH measurements*

pH_i was measured by the technique of distribution of the weak acid 7-[^{14}C]benzoic acid in the intracellular and extracellular spaces and calculated as in [8].

2.4. *DNA synthesis*

G_0-arrested cells were incubated for 24 h with [^{3}H]thymidine and labelled nuclei or [^{3}H]thymidine incorporated into acid-insoluble material were determined as described [23].

3. RESULTS

Fig.1A shows, as we reported [21], that the amiloride-sensitive Na^+ uptake, detected in CCL39 quiescent fibroblasts, is rapidly activated by purified growth factors: α-thrombin and insulin. In this experiment, $^{22}Na^+$ uptake was measured after imposing an outward-directed Na^+ gradient (15:1, in/out). Consequently, this reverse mode of functioning of the Na^+/H^+ exchange induces a net cytoplasmic acidification known to 'self-activate' the antiporter [15,22]. This feature accounts for the hyperbolic shape of the basal curve (non-stimulated cells).

When resting pH_i is measured under physiological conditions (inward-directed Na^+ gradient), addition of α-thrombin and insulin, to quiescent CCL39 cells, induces a rapid and persistent rise of 0.2–0.3 pH units [8] (fig.1C, left). In sharp contrast, PS120, a mutant derived from CCL39 cells specifically lacking Na^+/H^+ antiport activity [19], does not exhibit any basal amiloride-sensitive Na^+ flux (fig.1B). More importantly, in this mutant, α-thrombin and insulin fail to induce cytoplasmic alkalinization (fig.1C, right) and the amiloride-sensitive Na^+ influx (fig.1B). Therefore, the comparison of PS120 and its parent, regarding their rates of entry into S phase from a quiescent state, was judged as central in assessing the role of pH_i rise in mitogen action. The following experiments were conducted in bicarbonate-free medium. Experiments in the presence of CO_2 will be discussed later. When external pH (pH_o) is varied from 6.6 to 8.0, CCL39 cells begin to reinitiate DNA synthesis at pH_o 6.8. With increasing pH_o, the number of cells committed to enter the S phase increases progressively, peaks at pH_o

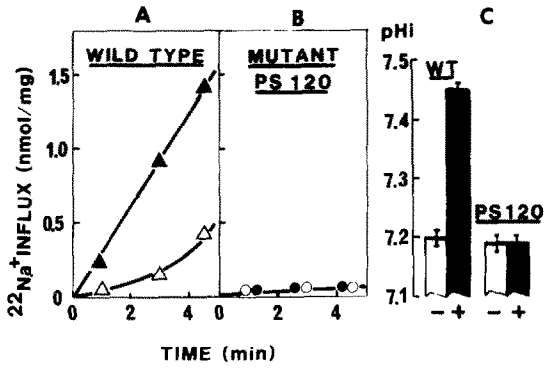

Fig.1. Amiloride-sensitive Na^+ uptake and cytoplasmic alkalinization of quiescent fibroblasts (wild-type and mutant PS120) in response to α-thrombin and insulin. (A,B) Amiloride-sensitive $^{22}Na^+$ uptake in wild-type cells (A) and in Na^+/H^+ antiport-deficient cells (B) in the absence (△, ○) or presence of α-thrombin and insulin (▲, ●). These uptakes were determined as described in section 2. When added, α-thrombin was at 1 NIH unit/ml and insulin at 10 μg/ml. Points represent mean values from duplicate dishes. (C) Intracellular pH of G_0-arrested wild-type (WT) and PS120 mutant cells before (empty bars) or after 15 min of stimulation with α-thrombin and insulin (solid bars). G_0-arrested cells were incubated for 30 min in DMEM buffered with 30 mM Hepes, pH 7.3. Then medium was replaced with the same medium containing 1 μCi/ml [^{14}C]benzoic acid and cells were incubated for 15 min in the presence (+) or absence (−) of α-thrombin and insulin. Intracellular pH was calculated as in [8].

7.4 and declines again at alkaline pH values (fig.2A). In contrast, the mutant cells display a DNA syntehsis response markedly shifted towards alkaline pH_o values and interestingly, both the lower pH_o limit and the optimal pH_o value are shifted by ~0.4 pH unis (fig.2A). Indeed, up to pH_o 7.2, α-thrombin and insulin fail to reinitiate DNA synthesis in PS120 cells (less than 0.1% labelled nuclei). In contrast, 50% labelled nuclei (80% of maximal stimulation) is observed at that pH_o (7.2) in the parental cells.

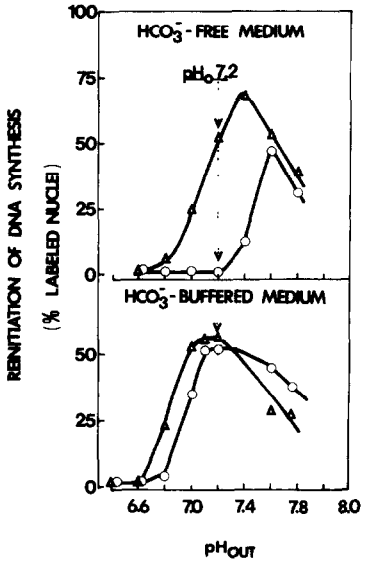

Fig.2. pH_o dependence for growth factor-induced DNA synthesis in quiescent CCL39 and PS120 mutant cells. Confluent cells in 35 mm dishes were incubated in the total absence of serum for 24 h in a 1:1 mixture of DMEM/Ham's F12 medium buffered with 25 mM HCO_3^-, pH 7.4 (atmosphere 5% CO_2/95% air). These G_0-arrested cells were then incubated for 24 h in the presence of α-thrombin (1 unit/ml) and insulin (10 μg/ml), 1 μCi/ml of [^{3}H]thymidine in DMEM buffered with 30 mM Mops (pH range 6.6–7.4) or EPPS (pH range 7.4–8.5). Incubations were in either the absence of CO_2 (A) or presence of 5% CO_2 (B). Cells were then processed for autoradiography as in [23]. Percentage of labelled nuclei was calculated from the examination of 3 independent fields containing more than 400 cells each. pH_o values plotted are those measured at the time of addition of growth factors. The change in pH_o did not exceed 0.15 pH unit after 24 h of growth factor action at the optimal pH_o. When cells were incubated in the presence of CO_2, pH_o values were measured after overnight equilibration in the CO_2 atmosphere.

The results of fig.2A were reproduced in 4 independent experiments in which we used either 10% FCS or α-thrombin and insulin as growth-promoting agents, or PS100 as an independent Na^+/H^+ antiporter-deficient clone [19].

We have seen (fig.1C) that addition of α-thrombin and insulin fail to induce cytoplasmic alkalinization in the Na^+/H^+ antiport-deficient mutant. As a direct consequence of this defect, over the range of external pH 6.6–8.2, pH_i remains 0.2–0.3 pH units more acidic in PS120 than in parental cells (fig.3A). From the established relationship $pH_o = f(pH_i)$ (fig.3A) and the results presented in fig.2A, we expressed the pH_i dependence for reinitiation of DNA synthesis as illustrated in fig.3B. This relation points out the following features: (i) mutant PS120 and parent cells have identical pH_i dependence for reinitiation of DNA synthesis; (ii) a marked pH_i threshold ~7.2 can be defined, below which growth factors

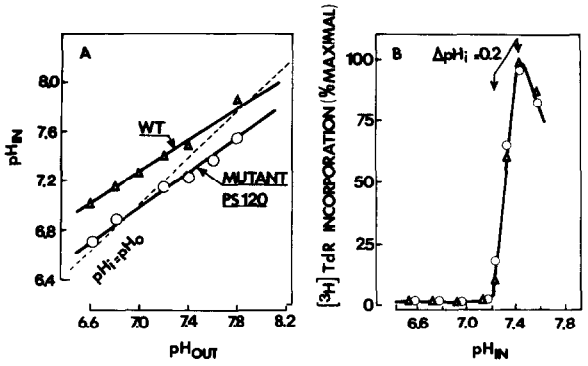

Fig.3. pH_i dependence for reinitiation of DNA synthesis and relationship between pH_{in} and pH_{out} in wild-type (Δ) and PS120 mutant cells (○). Cells were arrested as described in the legend to fig.2 and stimulated with α-thrombin (1 unit/ml) and 10 μg/ml insulin in an HCO_3^--free DME medium, buffered with either 30 mM Mops (pH_o 6.6–7.4) or 30 mM EPPS (pH_o 7.4–8.2). Prior to pH_i determination, cells were preequilibrated in the absence of growth factors at their respective external pH values for 60 min. Then the medium was replaced by the same one containing 1 μCi/ml of [^{14}C]benzoic acid and growth factors. pH_i (A) was determined after 10 min as described [8]. Each point is the mean value of 2 or 3 determinations. (B) [^{3}H]Thymidine incorporation into acid-insoluble material was measured after 24 h of stimulation with α-thrombin and insulin and the percent of maximal response was plotted against pH_i values determined in A.

fail to stimulate DNA synthesis; (iii) above this critical pH_i value, the rate of entry into S phase increases abruptly with pH_i. 90% of the maximal response is obtained within a pH_i variation of 0.2 pH units (fig.3B). This result reflects an extreme 'cooperative' effect of pH_i on the processes leading to DNA synthesis.

Two additional findings reinforce the idea that pH_i tightly controls reinitiation of DNA synthesis. First, we observed that the shift in pH_o dependence for DNA synthesis is almost completely abolished when bicarbonate is added to the medium of PS120 cells (cf. fig.2A and B). Regarding this observation we recently reported that hamster fibroblasts possess an Na^+-dependent Cl^-/HCO_3^- system involved in pH_i regulation [24,25] and more importantly that in HCO_3^--buffered medium the pH_i difference between PS120 and parental cells is suppressed [24]. Second, we selected a mutant, AS7, capable of growing in HCO_3^--free medium at pH_o 6.4, a non-permissive pH_o for the parental cells [19]. Therefore, we expected that AS7, which reinitiates DNA synthesis at lower pH (6.4 instead of 6.8), would maintain its pH_i at a more alkaline value than that observed in the parent cells. This was indeed the case (table 1). Over the range pH_o 6.4–7.2, the pH_i of AS7 is 0.2–0.3 pH units higher than in the wild-type cells. It is interesting to note that pH_o dependences for DNA synthesis have been altered in opposite directions in PS120 and AS7 mutant cells. These characteristics which result from genetic alteration of pH_i-regulating systems, demonstrate that pH_i dependence for DNA synthesis is an invariant feature of the cell.

Table 1

Comparison of resting pH_i and growth rate of CCL39 and its mutant derivative AS7

pH_o	Resting pH_i (growth factor-stimulated cells)		[^{3}H]TdR incorporation (% maximal response)	
	CCL39 (WT)	AS7	CCL39	AS7
6.4	6.75 (10)	6.95 (10)	1	8
6.8	7.1 (15)	7.35 (8)	5	60
7.2	7.3 (14)	7.60 (8)	65	95

pH_i was measured 15 min after growth factor stimulation of quiescent CCL39 (wild-type) and AS7 cells with α-thrombin and insulin [8]. pH_i values are the mean of the number of determinations indicated in parentheses. For [^{3}H]thymidine incorporation, cells arrested in G_0/G_1 were incubated for 24 h with growth factors in HCO_3^--free DME medium buffered either with 30 mM Mes or 30 mM Mops at the pH_o indicated

4. DISCUSSION

Among the questions which remain to be clarified is the identification of the pH_i-limited steps. One of the steps that we found highly pH_i dependent is the phosphorylation of ribosomal protein S6. Its phosphorylation, when compared in PS120 and its parent, follows their respective pH_o dependences for DNA synthesis [27]. In contrast, other early mitogen-stimulated events such as phosphoinositide breakdown (L'Allemain, G., Paris, S. and Pouysségur, J., in preparation), Na^+/H^+ antiport activation [8], phosphorylation of 41–43 kDa polypeptides [28] (Kohno, M. and Pouysségur, J., in preparation) and c-myc mRNA induction are not critically pH_i regulated. Another point of interest is to determine at what stage of the cell cycle and for how long a permissive pH_i (above 7.2) is required. Regarding this point, we have found that the increased pH_i (>7.2) is required throughout the G_0/G_1 period preceding DNA synthesis [27]. When the cells have reached the restriction point [29] (8 h after growth factor initiation) they can enter and progress into the S phase at much lower pH_i (Chambard, J.C. and Pouysségur, J., unpublished).

In conclusion, our results stress the fact that pH_i and therefore the growth factor activatable Na^+/H^+ antiporter plays a critical role in controlling the rate of cell progression from the G_0/G_1 state to S phase. This is the first genetic evidence linking the regulation of pH_i with the control of growth.

ACKNOWLEDGEMENTS

We thank Drs J.C. Chambard, M. Kohno and E. Van Obberghen-Schilling for critical reading of the manuscript and G. Clénet for secretarial assistance. This work was supported by grants from the Centre National de la Recherche Scientifique (LP 7300, ATP 136 and ASP 394), the Institut National de la Santé et de la Recherche

Médicale (CRE 84-2015), the Fondation pour la Recherche Médicale and the Association pour la Recherche contre le Cancer.

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