Monovalent cation-dependent reversible phosphorylation of a 40 S ribosomal subunit protein in growth-arrested *Tetrahymena*: correlation with changes in intracellular pH

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Phosphorylation and dephosphorylation of ribosomal protein S8 in starved *Tetrahymena* induced respectively by Na^+ and K^+ are accompanied by changes in intracellular pH which rises by about 0.8 pH units in cells starving in the presence of Na^+ (phosphorylation of S8) and falls by a little more than one pH unit after subsequent addition of K^+ (dephosphorylation of S8).

Ribosome; Protein; Phosphorylation; Intracellular pH

1. INTRODUCTION

Reversible phosphorylation of a small ribosomal subunit protein occurs in many eukaryotic organisms (proteins S6 in mammalian cells, tomato cells, *Dictyostelium discoideum, Mucor racemosus*, S1 in *Bombyx mori*, S3 in *Physarum polycephalum*, S10 in *Saccharomyces cerevisiae*; S8 [1], previously designated S7 [2], in *Tetrahymena thermophila*; for review see [3]). In growth-stimulated cells phosphorylation of this protein often shows evident temporal correlations with increases in the rate of protein synthesis and in intracellular pH [4,5]. Phosphorylation of S8 does not occur in growing *Tetrahymena* [6,7] but can be induced in starved cells (conditions not involving growth factors, hormones, and their receptors and ionic signals) by addition of appropriate cations (Na^+ but not K^+) to the starvation medium [8].

Here we show that cations which induce phosphorylation (Na^+) and dephosphorylation (K^+) of S8 in starved *Tetrahymena* also ihduce a concomitant rise (Na^+) or fall (K^+) in intracellular pH by mechanisms independent of a Na^+/H^+ antiport.

2. MATERIALS AND METHODS

2.1. *Reagents*

Choline chloride and amiloride were Sigma Grade from Sigma. Radioactive materials for measurement of intracellular pH were 5,5-dimethyl-[2-^{14}C]oxazolidine-2,4-dione (1.85 GBq/mmol) and D-[1-^{14}C]mannitol (1.85 GBq/mmol), obtained from New England Nuclear, and ^3H_2O (18.5 MBq/ml) supplied by CEA. All other products were as before [8].

2.2. *Buffers*

Starvation buffer: 10 mM Tris-HCl, pH 7.3, 1 mM $MgCl_2$. Protein extraction buffer: 50 mM Tris-acetate, pH 7, 50 mM NH_4Cl, 12 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 5 mM iodoacetamide.

2.3. *Organism and culture conditions*

Tetrahymena thermophila strain BIV was grown and cells were transferred to starvation media as in [8].

2.4. *Extraction of whole-cell protein*

For each analysis 5×10^6 cells were harvested from mid log cultures by centrifugation ($400 \times g$, 5 min, room temperature). All subsequent operations were at 0–4°C. Cell pellets were suspended in 0.5 ml of protein extraction buffer, 0.1 vol. of a 2.3% v/v solution of NP40 was added and the mixtures were vortexed gently. After complete cell lysis (~5 min, monitored by microscope), 0.5 vol. of protein extraction buffer was added; the mixtures were vortexed briefly, centrifuged ($10000 \times g$, 20 min) and the supernatants were transferred to tubes containing enough polyethylene glycol 6000 to give a final concentration of 12% (w/v). When polyethylene glycol was completely dissolved the mixtures were centrifuged ($10000 \times g$, 8 min), the supernatants were discarded, the pellets were suspended in 0.5 ml of protein extraction buffer, 2 vols of glacial acetic acid, and 0.1 vol. of 1 M $MgCl_2$ were added and the mixtures were stirred for 45 min. Insoluble material was removed by centrifugation ($10000 \times g$, 15 min) and proteins were precipitated from the supernatant by addition of 5 vols of acetone, collected by centrifugation dried in vacuo and dissolved in 8 M urea containing 5% (v/v) 2-mercaptoethanol and 3% (v/v) acetic acid.

2.5. *Two-dimensional electrophoresis*

The procedure of Zinker and Warner [9] modified slightly as in [8] was used.

2.6. *Determination of intracellular pH (pH_i)*

pH_i was measured as described by Gillies and Deamer [10].

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3. RESULTS

3.1. *Effect of cations other than Na^+ on the phosphorylation of protein S8 in starved* Tetrahymena thermophila

Na^+ but not K^+ induces phosphorylation of S8 in starved *Tetrahymena* and we have proposed that it may do so by provoking intracellular alkalinisation [8]. Results in table 1 compare the effects of Na^+ with those of other cations including organic amines which can increase pH_i by passive diffusion into the cell [11]. As can be seen, chlorides of lithium, choline, Tris, and triethanolamine all induce phosphorylation of S8 though with slower kinetics than NaCl. They also show that K^+ prevents phosphorylation of S8 if added with Na^+ to starved cells and moreover induces dephosphorylation of its mono- and diphosphorylated derivatives (S8′ and S8″, respectively) if added to cells preincubated in the presence of Na^+ to allow complete phosphorylation of S8. K^+ thus behaves as an inducer of dephosphorylation even in the presence of Na^+. We note here that transfer of cells containing the phosphorylated forms of S8 from Na^+ containing to Na^+ free starvation medium does not lead to their dephosphorylation presumably because of the presence of 10 mM Tris, a weak inducer of phosphorylation (Table I) in this medium.

Table 1

Effect of various cations on the phosphorylation of protein S8 in starved *Tetrahymena thermophila*

Pretreatment of cells	Additions to starvation buffer	Duration of incubation (h)	Estimated staining intensity		
			S8	S8′(*)	S8″(*)
(A) None	Na^+ 10 mM	5	−	+	+ +
		24	−	±	+ +
	Na^+ 40 mM	1	−	±	+ +
	Li^+ 20 mM	5(**)	+	+	+
	Choline 20 mM	5,24	+	+	+
	Triethanolamine 40 mM	5	+ +	−	−
		24	+	+ +	+
	Tris 50 mM	5	−	+ +	+
		24	−	+	+ +
	Na^+ 40 mM + K^+ 5 mM	1,5	+ +	−	−
		24	+	+ +	+
	Na^+ 40 mM + K^+ 20 mM	5,24	+ +	−	−
(B) Incubation in starvation buffer	Na^+ 40 mM + K^+ 5 mM	0.5	+ +	+	−
		1	+ +	±	−
	Na^+ 40 mM + K^+ 40 mM	0.5	+ +	−	−

* The mono- and diphosphorylated derivatives of S8 are designated S8′ and S8″, respectively

** In the presence of lithium cells do not survive beyond 5 h

(A) Total cell protein isolated from cells incubated at 31°C for 1, 5, and 24 h in starvation buffer containing the indicated additions was analysed by two-dimensional gel electrophoresis. Distributions of stained spots in the gels were similar to those in Fig. 1. The relative intensities of the spots containing proteins S8, S8′ and S8″ were estimated visually: + + intense; + weak; ± trace; − not visible. Although this is not evident in the photographs of sections of stained gels in Fig. 1 resolution of spots containing proteins S5 and S8″ is sufficient to allow confident evaluation of the relative intensities of staining of S8, S8′ and S8″. (B) As in A but cells were preincubated in the presence of 40 mM NaCl for 1 h at 31°C to allow complete phosphorylation of S8 before addition of K^+. Whole cell protein isolated after incubation of cells for 0.5 and 1 h in the presence of K^+ was analyzed

3.2. *Cation-induced changes in intracellular pH in starved* Tetrahymena

The opposite effects of Na^+ and K^+ on the phosphorylation of S8 suggested that they act indirectly by modifying the physiological state of starved cells rather than by direct effects on an S8-specific kinase/phosphatase system. Since phosphorylation of protein S6 of higher eukaryotes has been frequently correlated with changes in pH_i [4,5], and since organic amines which are known to provoke intracellular alkalinization in eukaryotic cells also induced phosphorylation of protein S8 in *Tetrahymena*, we examined the effects of Na^+ and K^+ on the pH_i of starved cells. Fig. 1 shows that the presence of Na^+ in the absence of K^+ leads to an increase in pH_i which is rapidly reversed by addition of K^+, and that these effects are accompanied respectively by phosphorylation and dephosphorylation of S8. In other experiments (results not shown), incubation of cells in starvation buffer containing 20 mM KCl or 20 mM KCl with 40 mM NaCl caused pH_i to decrease from 7.2 to about 6.8 in 60 min. Thus K^+ which prevents Na^+-induced phosphorylation of S8 also prevents Na^+-induced increase of pH_i. The counter effects of Na^+ and K^+, for normal cell growth [12] suggest that the other monovalent cations which induced phosphorylation of S8 would also behave like Na^+ towards pH_i.

3.3. *Effect of amiloride on changes in pH_i and in the state of phosphorylation of S8 in starved* Tetrahymena *before and after transfer of cells to growth medium*

In cultured mammalian cells phosphorylation of protein S6 and an increase in pH_i mediated by activation of the amiloride-sensitive Na^+/H^+ antiport often occur simultaneously following induction of division by treatment with growth factors or mitogens [4]. Therefore it was of interest to ask whether phosphorylation of S8 in starved *Tetrahymena* is mediated by activation of a Na^+/H^+ antiport. Since this antiport is sensitive to amiloride we examined the effect of 0.1–2 mM amiloride (concentrations >2 mM are lethal to *Tetrahymena*) on Na^+-induced intracellular alkalinization and phosphorylation of S8 in starved *Tetrahymena* and on the restoration of normal intracellular pH and

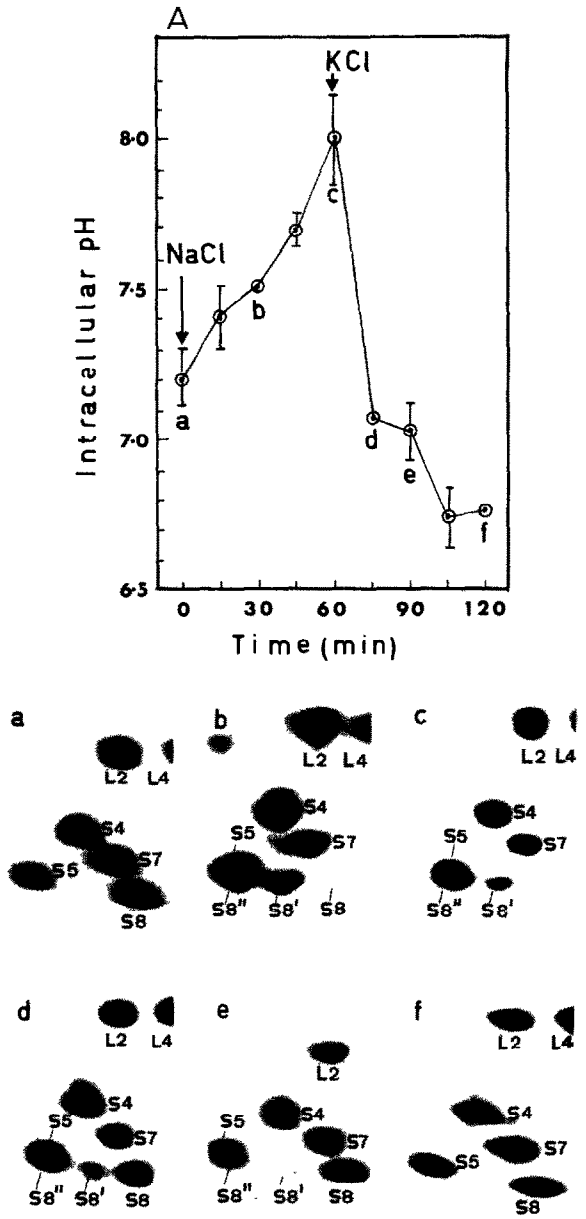

Fig. 1. Evolution of pH_i and of the state of phosphorylation of S8 in cells starving in the presence of Na^+ before and after addition of K^+. Exponentially growing cells were transferred to and incubated in starvation buffer at 31°C. After 1 h 40 mM NaCl was added (zero time) followed 60 min later by 25 mM KCl. Cells were harvested at 15 min intervals for pH_i determination and at time points a–f for protein analysis. (A) pH_i. (B) a–f, two-dimensional electrophoretic migration patterns of proteins extracted from cells harvested at time points a–f. Only the regions of gels containing S8, S8′ and S8″ and neighbouring spots are shown. In the gel system used no nonribosomal proteins and no 60S subunit proteins migrate in the vicinity of S8, S8′ and S8″. Protein numbering as in [1].

dephosphorylation of S8′ and S8″ in cells transferred from Na^+ containing starvation buffer to PPY growth medium. In all such experiments variation of pH_i and the rate and extent of phosphorylation and dephosphorylation of S8 were unaffected by the presence of amiloride (results not shown). Hallberg [13] also observed no effect of amiloride on dephosphorylation of S8 in cells transferred from Na^+ containing starvation buffer to growth medium.

4. DISCUSSION

A temporal correlation between phosphorylation of the small ribosomal subunit protein S6 and an increase in pH_i has been observed in several studies using growth-stimulated cells (fertilized sea urchin oocytes [4], hormone-treated *X. laevis* oocytes [14–16], serum or mitogen stimulated mammalian cells [17–19]). These observations have led to the suggestion that increased pH_i may cause phosphorylation of this protein in growth stimulated mammalian cells [20]. However recent studies have shown that an increase in pH_i is not necessary for phosphorylation of ribosomal protein S6 in hormone stimulated oocytes of *X. laevis* [21,22] or of the starfish *Marthasterias glacialis* [23]. It is therefore possible that in hormone or mitogen stimulated mammalian cells, increase in pH_i and phosphorylation of S6 may be independent manifestations of the effect of these stimuli on cell metabolism. The present study shows that, in *T. thermophila*, phosphorylation and dephosphorylation of ribosomal protein S8 can be induced in starved cells which, as we have previously shown [8,24], do not divide and display a slow net decline in their total RNA content, and a rapid reduction in their rate of protein synthesis. Under these conditions, which differ greatly from those pertaining in growth-stimulated cells, phosphorylation of protein S8 and an increase in pH_i are induced together by addition of Na^+ to the cell suspension medium, and the two effects are reversed together by subsequent addition of K^+. The close parallelism of these effects suggests that changes in pH_i and in the extent of phosphorylation of S8 may be related in this system. It has been shown in several studies with growth-stimulated cells that the increase in pH_i associated with phosphorylation of protein S6 is due to activation of the amiloride sensitive plasma membrane Na^+/H^+ antiport [18,19,25–28]. Although it has not been shown that *Tetrahymena* possesses this antiport it seemed of some interest to examine the effect of amiloride on variation of pH_i and phosphorylation/dephosphorylation of S8 in cells shifted to Tris-NaCl buffer and subsequently returned to nutrient medium. No effect of amiloride on either phenomenon was observed. It has also been reported recently [29] that although an Na^+/H^+ antiport is present, in murine splenic B lymphocytes, mitogen-induced intracellular alkalinization is not dependent on its operation. We conclude that if *Tetrahymena* possesses an amiloride-sensitive Na^+/H^+ antiport this system is not involved in the processes which regulate pH_i and the extent of phosphorylation of S8 in *Tetrahymena*. This is not surprising because

Tetrahymena does not require Na^+ for nutrient uptake and optimum growth in synthetic medium [12]. The present results show that reversible phosphorylation of S8 in starved *Tetrahymena* is strongly associated with variation in intracellular pH as reported in serum-stimulated Chinese hamster lung fibroblasts [5]; although in *Tetrahymena* variations in pH_i are not brought about by growth stimulation.

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