

Intracellular pH governs the subcellular distribution of hexokinase in a glioma cell line

Laurent MICCOLI*, Stéphane OUDARD*, Franck SUREAU†, Florence POIRSON*, Bernard DUTRILLAUX* and Marie-France POUPON*‡

* Laboratoire de Cytogénétique Moléculaire et Oncologie (UMR 147), CNRS-Institut Curie, Section de Recherche, 26 rue d'Ulm, 75231 Paris Cedex 05, France, and † Laboratoire de Physico-Chimie Biomoléculaire et Cellulaire (URA 2056), CNRS-Institut Curie, Section de Recherche, 11 rue Pierre et Marie Curie, 75231 Paris Cedex 05, France

Hexokinase plays a key role in regulating cell energy metabolism. Hexokinase is mainly particulate, bound to the mitochondrial outer membrane in brain and tumour cells. We hypothesized that the intracellular pH (pH_i) controls the intracellular distribution of hexokinase. Using the SNB-19 glioma cell line, pH_i variations were imposed by incubating cells in a high- K^+ medium at different pH values containing specific ionophores (nigericin and valinomycin), without affecting cell viability. Subcellular fractions of cell homogenates were analysed for hexokinase activity. Imposed pH_i changes were verified microspectrofluorimetrically by using the pH_i -sensitive probe SNARF-1-AM (seminaphthorhodofluor-1-acetoxymethyl ester). Imposition of an acidic pH_i for 30 min strongly decreased the particulate/total hexokinase ratio, from 63% in the control sample to 31%. Conversely, when a basic pH_i was imposed, the particulate/total hexokinase

ratio increased to 80%. The glycolytic parameters, namely lactate/pyruvate ratio, glucose 6-phosphate and ATP levels, were measured concomitantly. Lactate/pyruvate ratio and ATP level were both markedly decreased by acidic pH_i and increased by basic pH_i . Conversely, the glucose 6-phosphate level was increased by acidic pH_i and decreased by basic pH_i . To demonstrate that the change of hexokinase distribution was not due to altered metabolite levels of glycolysis, a pH_i was imposed for a 5 min incubation time. Modification of the hexokinase distribution was similar to that noted after a 30 min incubation, whereas metabolite levels of glycolysis were not affected. These results provide evidence that the intracellular distribution of hexokinase is highly sensitive to variations of the pH_i , and regulates hexokinase activity.

INTRODUCTION

Hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) is an ubiquitous enzyme found in yeast [1], plants [2] and mammalian tissues [3]. This enzyme catalyses the phosphorylation of glucose by MgATP to glucose 6-phosphate for its entry into glycolysis [4]. In mammals, there are four different iso-enzymes of hexokinase (termed hexokinase-I, -II, -III and -IV), which differ in their kinetic properties and in their cellular and subcellular distribution [3,5–8]. Hexokinase type I isoenzyme, the major isoform of the enzyme in brain, is either free in the cytosol or bound to mitochondria [9,10].

Mitochondrial binding of hexokinase is altered in pathological conditions, such as in cancer, diabetes mellitus, brain hypoxia and ischaemia, and congenital metabolic disorders [11,12]. High levels of hexokinase bound to mitochondria are observed in a number of malignant and transformed cell lines [13–16]; however, the precise role of mitochondria-hexokinase interactions and their metabolic impact are not well understood. The enzymic activity of mitochondria-bound hexokinase is higher than that of the free form [17], and hexokinase activity extracted from tumour cells exceeds that of all normal cells studied [18]. When associated with mitochondria, the affinity of hexokinase for ATP is increased [19,20] and feedback inhibition by glucose 6-phosphate (an important regulator of hexokinase in normal cells) is almost completely abolished [13,15,21]. Physiological concentrations of glucose 6-phosphate have been shown to solubilize the mitochondria-bound hexokinase from both tumour cell [21] and rat brain [17]. Hexokinase is bound to the mitochondrial outer

membrane [22] at the level of a porin, also called VDAC (voltage-dependent anion channel) [23,24], by its N-terminal sequence, which plays the role of an anchorage peptide [25]. This suggests that mitochondria-bound hexokinase uses freshly mitochondrially generated ATP extruded through the VDAC and favours the delivery of ADP generated by hexokinase activity to oxidative phosphorylation [13,19,26]. Moreover, the enhanced glucose 6-phosphate production associated with hexokinase binding tends to maintain a high glycolytic energy-production rate, thereby contributing to the support of active biosynthesis of the nucleic acids and phospholipids required for the rapid growth of tumour cells, and evidence suggests that in these cells hexokinase activity may control rates of glycolysis [27,28].

Very little is at present known about the mechanism anchoring hexokinase to mitochondria *in vivo*. The high levels of hexokinase binding observed in the brain a few seconds after experimental ischaemia in the chicken [29] suggest wide availability of free hexokinase for binding to mitochondria in relationship with metabolic stress.

All cellular processes may be affected by the intracellular pH (pH_i), whose variations seem to be important in controlling the cell cycle and the proliferative capacity of cells [30]. Several processes, such as glutaminolysis, aerobic and/or anaerobic glycolysis, and ATP hydrolysis, are responsible for the production of H^+ ions [31]. Lactate- H^+ are released from the cells, creating an acidic extracellular pH (pH_o) [32]. The pH_i of most tumours and normal tissues is near neutrality or slightly alkaline [32,33]. Mechanisms regulating pH_i are essential for cell survival in an acidic environment [34,35].

Abbreviations used: VDAC, voltage-dependent anion channel; pH_i , intracellular pH; pH_o , extracellular pH; DMEM, Dulbecco's modified Eagle's medium; SNARF-1-AM, seminaphthorhodofluor-1-acetoxymethyl ester; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; mHK, mitochondrially bound hexokinase.

‡ To whom correspondence should be addressed.

We hypothesized that variation of the pH_i could be a critical regulator of hexokinase-mitochondria interactions, and thus of glycolysis. pH_i changes, monitored by means of a fluorescent pH probe, were imposed on a glioma cell line in culture, by using specific ionophores. Subsequent modifications in the activity of mitochondria-bound hexokinase were investigated, and their relationships with pH_i values were demonstrated. This pH_i effect on hexokinase was then analysed in terms of lactate/pyruvate ratios, glucose 6-phosphate and ATP levels measured in the same samples.

MATERIALS AND METHODS

Cell culture

The glioma-derived cell line SNB-19 [36] was cultivated in DMEM (Dulbecco's modified Eagle's medium) (Sigma, St. Louis, MO, U.S.A.) containing 25 mM glucose at pH 7.4 and supplemented with 10% (v/v) fetal-calf serum (Dutscher, Brumath, France), 2 mM L-glutamine, 50 units/ml penicillin and 50 μ g/ml streptomycin, and 10 ng/ml epidermal growth factor (Sigma). Cells were maintained at 37 °C, with air/CO₂ (19:1), 100% humidity, and harvested for passage when they reached confluence. Cell viability was estimated by Trypan Blue dye exclusion and was > 95% at the start of each experiment.

Alterations of pH_i

pH_i was altered as reported by Thomas et al. [37]. Sub-confluent cells were trypsin-treated (trypsin-EDTA 0.5 mg/ml) (Sigma), suspended in a large volume of DMEM and centrifuged for 5 min at 700 *g*. The pellet was then resuspended in a small volume of PBS, pH 7.4 (Sigma). The cells were counted with a Coulter Counter ZM (Coultronics France SA, Magency, France), and 50×10^6 cells were distributed into 50 ml conical tubes. A high-K⁺ buffer (10 mM Hepes, 130 mM KCl, 20 mM NaCl, 1 mM CaCl₂, 1 mM KH₂PO₄, 0.5 mM MgSO₄ and 5 mM glucose), previously adjusted to different pH values (6.7, 7.4 and 8, obtained by the addition of small amounts of conc. KOH or HCl solution), was added to the cell suspensions, and incubations were carried out for 5 or 30 min at 37 °C. Stock solutions of ionophores (Sigma) were prepared in ethanol: nigericin at 2 mg/ml and valinomycin at 10 mM. Nigericin (1 μ g/ml) and valinomycin (5 μ M) were added to the cell suspensions, enabling a rapid equilibration of pH_o and pH_i . After incubation at the indicated time and temperature, the cells were centrifuged at 700 *g* for 5 min at 4 °C, the supernatants were discarded, and the pellets were rapidly frozen in liquid nitrogen and stored at -80 °C. By using the specific fluorescent probes SNARF-1-AM (seminaphthorhodafuor-1-acetoxymethyl ester) and JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) (Molecular Probes, Eugene, OR, U.S.A.), parallel experiments were run to monitor both pH_i changes and mitochondrial membrane potential.

Preparation of hexokinase fractions

All steps were performed at 4 °C. The pellets were rapidly thawed and immediately resuspended and homogenized in an Potter homogenizer in a 10-fold excess of extraction buffer (10 mM Tris/HCl, pH 7.7, 0.25 mM sucrose, 0.5 mM dithiothreitol, 1 mM aminohexanoic acid and 1 mM PMSF). The homogenate was centrifuged for 15 min at 800 *g* to remove nuclei and cell debris. The pellet was washed once with extraction buffer, and the 800 *g* supernatants were pooled as the 'total hexokinase fraction' and further prepared as described by Sprengers et al. [38] with modifications. The 800 *g* supernatant pool was centri-

fuged for 15 min at 48000 *g*. The 48000 *g* supernatant was referred to as the 'cytosolic fraction'. The 48000 *g* pellet was washed once with extraction buffer. The hexokinase activity in the final pellet was referred to as the 'mitochondria-bound fraction'. The final pellet was resuspended in the extraction buffer supplemented with 10 mM MgCl₂ to prevent spontaneous solubilization.

Hexokinase activity assay

Enzyme activity was determined in a system coupled with glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (Sigma). A final volume of 1 ml of assay medium contained: 100 mM Tris/HCl (pH 8), 10 mM glucose, 0.4 mM NADP⁺ (Sigma), 10 mM MgCl₂, 5 mM ATP and 0.15 unit of glucose-6-phosphate dehydrogenase. The reaction was started by addition of the extract sample and monitored at 340 nm in an Uvikon 830 CL spectrophotometer equipped with a Kontron cell changer 900 (Basel, Switzerland). One unit is defined as the amount of enzyme converting 1 μ mol of substrate into 1 μ mol of product per min at 30 °C. Before determination of the hexokinase activity in subcellular fractions, all samples were incubated on ice with 0.5% Triton X-100 for 20 min. The total recovery of hexokinase activity was always > 90%. The protein content was measured by the BCA protein assay (Pierce Chemical Co., Rockford, IL, U.S.A.) with BSA as the standard.

Glucose 6-phosphate, ATP, pyruvate and lactate determinations

Glucose 6-phosphate was assayed by the method of Lowry et al. [39]. The ATP pool was determined with the ATP bioluminescent assay kit (Sigma) by means of the luciferin-luciferase reaction [40], using a Lumac Biocounter M1500 luminometer (Lumac, Landgraaf, The Netherlands). Lactate and pyruvate were assayed enzymically [41] with a Cobas Fara centrifugal automatic analyser (Diagnostic Roche, Ile-de-la-Jatte, France).

Laser microspectrofluorimetry of SNARF-1-stained cells

SNARF-1-AM is a rhodamine-derivative dye that generates two pH -sensitive bands in its fluorescence emission spectrum, thereby enabling pH_i determination by microspectroscopy under defined staining conditions [42]. The probe was dissolved in DMSO (Sigma) at 1 mM and used at a final concentration of 10 μ M (1% DMSO). Fluorescence emission spectra from single living cells loaded with SNARF-1-AM were then obtained with our microspectrofluorimeter prototype (URA 2052, Institut Curie, Paris, France) [43]. The 514.5 nm line of an argon laser (model 2025; Spectra Physics, Mountain View, CA, U.S.A.) was used for excitation. The laser beam was focused on the sample through the microscope objective ($\times 63$ Zeiss Neofluar water-immersion objective, numerical aperture = 1.2) on a 0.5 μ m-diameter circular spot. The excitation power was decreased to less than 0.1 μ W by neutral optical-density filters in order to avoid any photodamage of the sample. Fluorescence emission spectra were recorded in the range 530–790 nm on a 1024 diode-intensified optical multichannel analyser (Princeton Instruments, Princeton, NJ, U.S.A.). Data were stored and processed on a 80386 IBM PS/2 microcomputer with the Jobin-Yvon 'Enhanced Prism' software (Longjumeau, France).

Fluorescence microscopy of JC-1-stained cells

JC-1 is a positively charged carbocyanine dye that can be exploited as a potential-sensitive probe of the mitochondrial membrane in living cells. The probe was dissolved in DMSO at 1 mg/ml and used at a final concentration of 10 mg/ml (1%

DMSO). Once JC-1 exceeds a critical concentration, it forms so-called J-aggregates (emission 590 nm), whose red fluorescence can be easily distinguished from the green fluorescence of the monomer (emission 540 nm) [44]. JC-1 accumulation in mitochondria increases linearly with membrane potential, resulting in a linear increase in red fluorescence. Thus the colour of the dye changes reversibly from green to red as a function of the membrane potential of energized mitochondria. This feature has been used to assess, by fluorescence microscopical observations, the possible alteration of the mitochondrial membrane potential caused by various cell treatments. Conditions for cell staining and fluorescence observation under the microscope were as described in [45]: pretreated cells were incubated with JC-1 for 10 min and washed before fluorescence observation.

Determination of the respiratory control ratio

Organelles were isolated from SNB-19 cells by differential centrifugation as described by Lai and Clark [46], with minor modifications. The cells (250×10^6) were ground with 70 strokes of a tight-fitting glass pestle in a Potter–Elvehjem glass homogenizer. Oxygen consumption was recorded with a Clark-type oxygen electrode [47]. Mitochondria (0.8 mg/ml) were incubated in a total volume of 0.5 ml of respiratory buffer (75 mM mannitol, 25 mM sucrose, 100 mM KCl, 10 mM Trizma phosphate, 50 μ M K_2 EDTA, pH 7.4) at 25 °C, using a Gilson Oxygraph (model 5/6). Succinate (10 mM) and the NAD⁺-linked respiratory substrate, such as glutamate (10 mM), each added with 2.5 mM malate, showed respiratory control ratios of 10 and 3 respectively. The State-3/State-4 transitions for oxidation of these substrates were induced with small additions of ADP (137 μ M).

RESULTS

Modification of pH_i

Glioma cells were suspended in a high-K⁺ buffer at various pH values, and rapid equilibration of pH_i and pH_o was obtained by adding specific ionophores (nigericin and valinomycin) to the suspension buffer [37]. Actual pH_i modifications were monitored with the specific pH probe SNARF-1-AM, by measurements of the fluorescence intensities (If) of two bands (635 and 592 nm) of its emission spectrum and calculation of the ratio $R = If_{635}/If_{592}$. In the presence of both ionophores (Figure 1A), the mean R values, obtained from the analysis of 20 cells, increased from 0.48 at pH_o 6 to 0.77 at pH_o 8. Typical distributions of R values at pH_o 6, 7.4 and 8 are shown in Figure 1(B). All distribution patterns showed a relatively good homogeneity in all experiments. Similar values of imposed pH_i changes were obtained for treatment periods of 5 to 60 min. Similar experiments using valinomycin alone in high-K⁺ buffer (Figure 1A) gave the same mean R ratio as that found with both ionophores at pH_o 6 (R = 0.48), but it led to an acidification of the cell in physiological pH_o 7.4 buffer (R = 0.55) and to a pH_i close to the physiological pH for a pH_o of 8 (R = 0.62). Finally, the R obtained at pH 7.4 without any ionophores was quite similar to that obtained in presence of both ionophores (0.64 and 0.63 respectively). Therefore it could be supposed that, under the usual culture conditions, the pH_i of SNB-19 cells is very close to 7.4.

pH_i-dependence of hexokinase subcellular distribution

Cells, ionophore-treated or not, were subjected to imposed pH_i for 5 and 30 min, then fractionated, and hexokinase activity in the subcellular fractions was measured. In the absence of ionophore, the distribution of hexokinase activity between cyto-

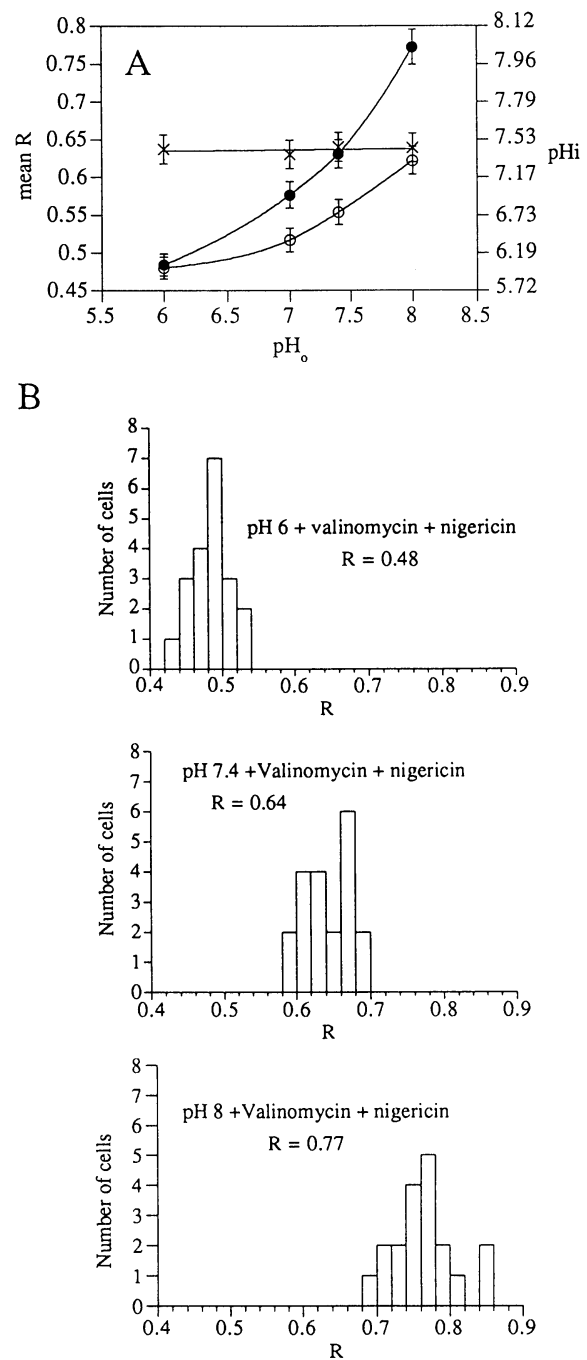


Figure 1 Effect of imposed pH_i changes on a glioma cell line treated with specific ionophores

(A) The ratio R of SNARF-1 probe fluorescence intensities (If) and corresponding pH_i versus pH_o of the high-K⁺ buffer: x, control cells; ●, cells treated with both valinomycin and nigericin; ○, cells treated with valinomycin alone. For details see the Materials and methods section. When valinomycin was used alone, R values obtained were lower, suggesting that valinomycin is not sufficient to equilibrate pH_o and pH_i. (B) Typical distribution of the R value obtained in the presence of valinomycin plus nigericin at the indicated pH_o (analysis of 20 single cells). All distribution patterns exhibited relatively good homogeneity, regardless of the experiment, with R increasing as a function of increasing pH_o. The values are means \pm S.D. of three separate determinations made in three separate experiments.

sol and mitochondrial fractions was not affected in high-K⁺ buffer regardless of the pH_o: $63 \pm 4\%$ of mitochondria-bound hexokinase. In contrast, it was altered for cells treated with

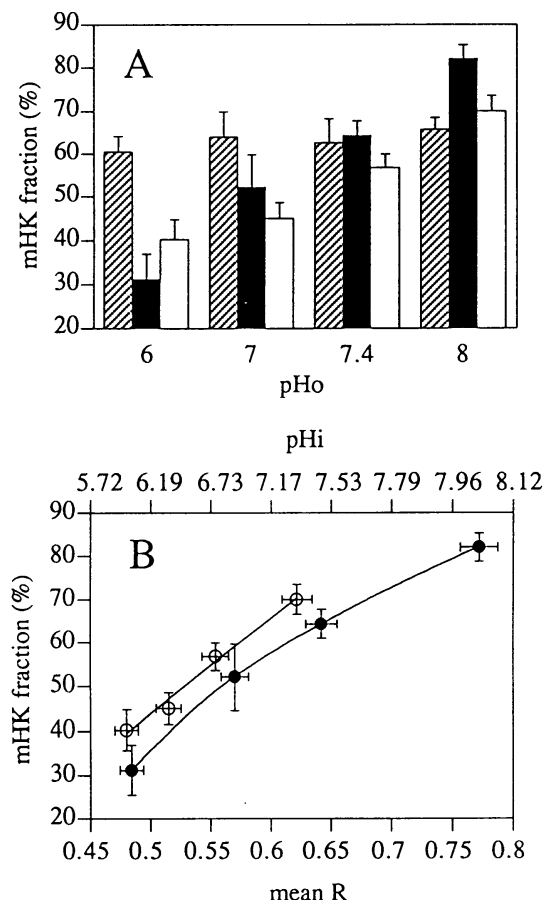


Figure 2 pH_i -dependence of the subcellular distribution of hexokinase

Hexokinase activity was measured in subcellular fractions obtained after cells were treated for 5 or 30 min at different pH_o values. For details see the Materials and Methods section. (A) The activity in the mitochondria-bound hexokinase fraction (mHK fraction) is represented as a function of the pH_o of the high- K^+ buffer. In the absence of ionophore (hatched bars), the hexokinase distribution was not affected at any pH_o , whereas it was markedly changed in cells treated with both ionophores (black bars), and to a lesser extent when valinomycin was used alone (white bars) at basic pH_o . (B) The same values for the mHK fraction are represented here as a function of the mean R ratio (see Figure 1A) and the corresponding pH_i : ●, cells treated with both ionophores; ○, cells treated with valinomycin alone. These curves demonstrate that the changes in the enzymic activity in the mHK fraction strongly reflects the imposed pH_i changes. The values are means \pm S.D. of determinations made in three separate experiments.

valinomycin alone or in combination with nigericin when the pH_o was modified (Figure 2A). In cells exposed to both ionophores, hexokinase activity associated with the mitochondrial fraction ranged from $31.2 \pm 5.7\%$ to $82 \pm 3.2\%$ for pH_o 6 and 8, respectively. When cells were incubated with valinomycin alone in high- K^+ buffer, modification of the pH_o from 6 to 8 changed the hexokinase distribution less than after treatment with both ionophores, since the fraction of mitochondria-bound hexokinase ranged from $40.2 \pm 4.5\%$ to $70.3 \pm 3.5\%$ respectively. Similar results were obtained with both treatment periods (5 and 30 min). Finally, the activity of particulate hexokinase directly reflected the imposed pH_i changes, regardless of treatment (Figure 2B), whether with valinomycin alone or both ionophores.

Mitochondrial membrane-potential control experiments

Effects of high K^+ , valinomycin and nigericin on the mitochondrial membrane potential of treated cells were monitored by

fluorescence microscopy of JC-1-stained cells. The red fluorescence of the J-aggregates was observed when cells were incubated in high- K^+ buffer without ionophores or in the presence of nigericin, indicating that, in this case, the mitochondrial membrane potential is not affected. On the other hand, the green fluorescence of JC-1 monomers was only observed when valinomycin was added to the cell medium, indicating a lack of membrane potential, in agreement with previously published results [45].

Glycolysis parameters

In parallel assays, the levels of glucose 6-phosphate, pyruvate, lactate and ATP were measured in cells incubated in high- K^+ buffer, treated or not with both ionophores at various pH_o values during 5 or 30 min, in order to assess the possible cause of the observed shift in hexokinase distribution. Lactate/pyruvate ratios in cells treated with both ionophores for 30 min increased with increasing pH (3.6- and 2.3-fold from pH_o 6 and 7 to 8, respectively), whereas those of the untreated controls remained relatively stable at all the pH_o values (Table 1). The ATP content of treated cells was sharply decreased (> 13 -fold) when an acidic pH_i was imposed, and decreased 2-fold when a pH_i of 7 was imposed, but levels comparable with those of controls were obtained at pH_o 7.4 and 8. Afterwards, the ATP contents in all samples increased with increasing basicity. The glucose 6-phosphate level in cells treated with both ionophores for 30 min rose when pH_i values of 6 and 7 were imposed, and decreased when a pH_i of 8 was imposed, whereas those of the untreated controls remained relatively stable at all the pH_o values (Table 1).

When cells were treated for 5 min, lactate/pyruvate ratios, ATP and glucose 6-phosphate levels at each pH_o value were comparable with those of controls.

DISCUSSION

Our basic hypothesis was that the process of hexokinase binding to mitochondria was dependent on pH_i . By using a glioma cell line, previously characterized in our laboratory as having a very stable high particulate hexokinase level ($63 \pm 4\%$), protocols of cell treatment suitable to induce pH_i changes from 6 to 8 were devised. The subcellular distribution of hexokinase and glycolysis parameters were evaluated as a function of various imposed pH_i values.

According to the method developed by Thomas et al. [37], the pH_i can be modified for 1 h without affecting cell viability, when cells are placed in a high- K^+ buffer containing two ionophores, nigericin and valinomycin. The K^+ concentration of the iso-osmotic buffer used for the experiments was set at the estimated intracellular K^+ concentration [48]. Moreover, valinomycin (an ionophore which allows equilibration of the intracellular and extracellular K^+ concentrations) was added in order to prevent the residual transmembrane K^+ gradient from affecting pH equilibration. In the absence of a transmembrane K^+ gradient, the addition of nigericin (a K^+/H^+ antiport) causes rapid equilibration of pH_i and pH_o . In the absence of ionophores, the pH_i was not altered by the modification of pH_o , and a 5 min incubation with both ionophores in high- K^+ buffer was sufficient to equilibrate pH_i and pH_o [37], whereas treatment with valinomycin alone led to intracellular acidification as compared with pH_o .

It should be noted that, regardless of the means used to modify the pH_i , the activity of the mitochondria-bound hexokinase fraction was markedly increased ($31.2 \pm 5.7\%$ to $82 \pm 3.2\%$ for

Table 1 Cell glycolysis parameters at the imposed pH_i

Glioma cells were incubated in a high- K^+ buffer containing both ionophores at various pH_o values and compared with the control cells treated under the same conditions without ionophores. Lactate/pyruvate ratios, glucose 6-phosphate and ATP levels were determined after 5 min and 30 min incubations. The results are means \pm S.D. of determinations made in three separate experiments.

pH_o	Glucose 6-phosphate (nmol/mg of protein)		Lactate/pyruvate ratio		ATP (nmol/mg of protein)		
	30 min	5 min	30 min	5 min	30 min	5 min	
pH_o 6	Treatment	0.92 \pm 0.07	0.58 \pm 0.04	4.5 \pm 0.9	9.0 \pm 1.5	2.3 \pm 0.3	20.6 \pm 2.5
	Control	0.60 \pm 0.06	0.63 \pm 0.05	11.1 \pm 2.5	10.1 \pm 2.0	25.7 \pm 3.2	22.7 \pm 2.1
pH_o 7	Treatment	0.75 \pm 0.03	0.56 \pm 0.04	7.1 \pm 0.8	10.0 \pm 1.3	18.2 \pm 3.0	24.2 \pm 2.6
	Control	0.51 \pm 0.06	0.53 \pm 0.07	12.9 \pm 2.1	12.5 \pm 1.8	29.3 \pm 2.8	28.2 \pm 2.3
pH_o 7.4	Treatment	0.59 \pm 0.05	0.62 \pm 0.06	14.3 \pm 2.8	14.0 \pm 2.6	31.4 \pm 3.5	29.7 \pm 2.9
	Control	0.57 \pm 0.07	0.66 \pm 0.06	13.5 \pm 4.2	14.1 \pm 3.1	34.2 \pm 5.2	29.1 \pm 4.5
pH_o 8	Treatment	0.29 \pm 0.04	0.52 \pm 0.06	16.2 \pm 2.5	12.7 \pm 3.2	34.4 \pm 4.3	31.9 \pm 3.7
	Control	0.60 \pm 0.06	0.58 \pm 0.05	11.1 \pm 2.0	12.0 \pm 2.8	32.5 \pm 4.8	25.3 \pm 3.4

pH_i 6 and 8 respectively), and a clear relationship was observed between the level of particulate hexokinase and the imposed pH_i . It must be emphasized that the levels of mitochondria-bound hexokinase obtained were not dependent on the length of treatment (5 or 30 min).

Nevertheless, although treatment of the cells with valinomycin plus nigericin induced the required modifications of pH_i , it also led to a breakdown of the mitochondrial membrane potential. However, this membrane depolarization did not affect the intracellular distribution of hexokinase, since the level of particulate hexokinase obtained at pH_o 7.4 was the same in the presence or absence of both ionophores. Thus a possible relationship between the particulate hexokinase level and mitochondrial-membrane-potential alteration was excluded.

It has been clearly established that the pH_i is an important regulator of several enzymic activities [30]. Our results showed that some critical parameters of aerobic glycolysis, such as the lactate/pyruvate ratio, glucose 6-phosphate and ATP levels, were modified at various imposed pH_i values, and these changes may be attributed to either particulate hexokinase, or pH_i alone, or a combination of these factors. Specific inhibition by glucose 6-phosphate is known to be the main regulator of hexokinase activity, and this substance has been shown to solubilize the bound hexokinase from mitochondria [49], inducing a conformational change of the enzyme and modulating its interaction with the mitochondrial membrane [50]. Reversible interactions, less specific than that of glucose 6-phosphate, between hexokinase and mitochondria could control its activity [10].

The changes in glucose 6-phosphate level, measured when the pH_i was imposed for 30 min, would lead one to predict an increase in bound hexokinase at basic pH_i and a decrease at acidic pH_i ; both changes were observed in this work. This suggests that changes in pH_i extensively affect cellular glucose 6-phosphate levels, with consequent modifications in hexokinase-mitochondria binding. Nevertheless, although imposed pH_i changes for a 5 min incubation time induced a similar shift in hexokinase distribution, it did not lead to changes in glucose 6-phosphate level, since the level of this metabolite, well known to be the main regulator of hexokinase activity, remained approximately stable as the controls. Kyriazi and Basford [51] examined the distribution of brain hexokinase *in vivo* during periods of ischaemia after rats were killed. They observed that

the levels of glucose 6-phosphate and glucose 1,6-bisphosphate fell, whereas a slight decrease in particulate hexokinase was obtained. They suggest that the failure of the shift in hexokinase distribution to correlate with levels of the usual effectors may be explained by the opposing action of increased acidity, resulting from hypoxia [51,52]. Indeed, increased acidity has been reported to cause solubilization of bound hexokinase *in vivo* [52], and also results in increased glucose 6-phosphate levels in brain [53]. In isolated rat brain mitochondria, Felgner and Wilson [49] found increased solubilization of particulate hexokinase as the pH was increased in presence of glucose 6-phosphate, whereas pH had only modest effects on solubilization of the enzyme in the absence of glucose 6-phosphate. However, these same authors found that an increased acidity promotes solubilization of bound hexokinase and allows glucose 6-phosphate-induced solubilization in high-ionic-strength medium (0.2 M KCl or NaCl) [49], ionic concentrations not far from those existing in the cytoplasm [48]. It could be postulated that the distribution of hexokinase by pH in intact cells might involve factors, such as enzymes that are not active (dilution, lack of cofactor such as ATP) in cell-free systems.

Lactate/pyruvate ratio and ATP level were much lower at the imposed pH_i of 6, as reported previously [54]. A low pH_i inactivates phosphofructokinase, a rate-limiting enzyme of glycolysis [55], and halts respiratory activity [56]. At basic pH_i , lactate/pyruvate ratio and ATP level were high; it is also known that phosphofructokinase activity increases greatly, as do glycolysis and the energy charge of cells, in response to a pH increase even for small pH intervals [57]. It must be noted that all the metabolic changes recorded at 30 min were not observed after 5 min of induced pH_i changes, suggesting that they are a consequence of effects of pH_i on cell metabolism.

In contrast with normal cells, tumour cells, and especially the glioma cells like those studied here (which exhibit elevated amounts of mitochondria-bound hexokinase and a high rate of glycolysis), are mainly dependent on the glycolysis pathway for their ATP requirements [58–60]. The pH_i -sensitivity of particulate hexokinase, one of the most important enzymes for maintaining a high glycolytic energy-production rate, has been demonstrated here, even though the conditions used in this study exceed physiological ones. This dependency suggests functional coupling to the inner mitochondrial compartment, giving to these cells a

metabolic advantage. It may constitute a potential approach for anti-tumour therapy to decrease their energy metabolism [58]. Our findings revealed that the subcellular distribution of hexokinase is strongly dependent on the pH_i , and that changes in this distribution occur as a fast and dynamic process, and may be related to a pH-induced modification of the protein conformation. It is suggested that the pH_i , by maintaining hexokinase bound to mitochondria, may play a determinant role in regulating this enzyme's activity.

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