Interactions Between Intracellular Chloride Concentrations, Intracellular pH and Energetic Status in Rat Lactotrope Cells in Primary Culture

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Abstract. Rat lactotrope cells in primary cultures have a higher intracellular Cl^- concentration ($[Cl^-]_i$) than that predicted by a passive distribution across the membrane. This suggests that active cellular mechanisms ensure this ionic equilibrium. In this study, we examined the interactions between pH_i, $[Cl^-]_i$ regulation and cell energetics. We analyzed: 1. the interactions between extracellular Cl^- concentrations, $[Cl^-]_i$ and cellular energy; 2. the influence of $[Cl^-]_i$ on respiratory chain function; 3. the correlation with glycolysis and; 4. the role played by pH_i in these cellular mechanisms. We show that low $[Cl^-]_i$ decreases ATP cell content, ATP/ADP ratio and modify phosphorylative oxidations. ATP production is rather due to the anaerobic pathway of the glucose metabolism than the aerobic one and depends also on other metabolic substrates among which glutamine probably has a special role. Finally, pH_i appears as a determinant in the balance between aerobic and anaerobic pathways. These results are discussed in relation to the role of Cl^- in normal and pathological (effect of hypoxia on mature and immature neurons) cell situations.

Key words: Rat lactotrope cells — Cell energetics — Aerobic and anaerobic pathways — pH_i — $[Cl^-]_i$

Introduction

Chloride ions (Cl^-) play a crucial role in rat lactotrope cell physiology. These cells in fact possess higher intracellular Cl^- concentrations ($[Cl^-]_i$), than those which

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would result from the electrochemical equilibrium (Garcia et al. 1997a,b), suggesting that they have developed mechanisms to insure uphill Cl^- entry. Beside ionic channels, Ca^{2+}-dependent chloride conductance (Sartor et al. 1992; Fahmi et al. 1995) and gamma-aminobutyric acid (GABA)-activated chloride channel (Lorsignol et al. 1994), we have also described the anionic exchangers AE2 and AE3 (Garcia et al. 1997c) and demonstrated the existence of Cl^- stores in mitochondria (Garcia et al. 1997b). In addition, a heterogeneity of lactotrope cell physiology has been reported in previous papers (Zhang et al. 1990; Lledo et al. 1991). In the accompanying paper we provide evidence to demonstrate that rat lactotrope cells are able to by-pass the low Cl^- concentration of the patch pipette by activating energy-dependent physiological mechanisms (Sartor et al. 2004). In this study, the possible link between cell energetic status and $[Cl^-]_i$ regulation is analyzed. Little quantitative data concerning the interaction between energy metabolism and cellular ionic homeostasis are available, whereas this type of interaction is likely to play a primordial role in eucariotic cell biology. Some previous reports have examined the cellular mechanisms sustaining cellular energy production in rat lactotroph cells (Vannucci and Hawkins 1983) or demonstrated an interaction between $[Cl^-]_i$ and the activity of the ATP-dependent K^+ channel in insulinoma cells (DeWeille and Lazdunski 1990). Recent studies have emphasized the specific effect of the ionic environment on respiratory chain functioning (Devin et al. 1997). Thus, it appears that more information about Cl^- homeostasis and cell energetic relationships in rat lactotrope cells is necessary. The aim of this work is to analyze interactions between intracellular pH (pH_i), $[Cl^-]_i$ regulation, and cell energetics.

Materials and Methods

Cell cultures

Pituitary glands were obtained from lactating female rats a few days after separation from the pups. The anterior lobe was dissected free of the intermediate and posterior lobes, cut into small fragments treated by 0.1% trypsin (Seromed, Strasbourg, France) and cells were plated on 30 mm glass coverslips (Sartor et al. 2003).

In order to determine the nucleotide content (ATP and ADP), cells were also seeded in 24 wells plates ($\sim 10^5$ cells *per* well) to be used after 4 days of culture. Culture medium was changed for the last 24 h of culture.

For respiration studies, cells were grown on gelatin beads (Cultispher-GL, Percell Biolytica AB, Sweden), (Martin et al. 1998). After seeding (3 to 5 million cells for 20 mg of beads in 4 ml of medium), they were introduced into 15 ml conic tubes, gassed with a sterile air-CO_2 mixture (85–15%), continuously agitated on a roller for 24 h and then transfered to 35 mm Petri dishes. Media were changed on the first and fourth days. Respiration studies were done on the fifth day of culture.

Microspectrofluorimetry

Intracellular Cl^- studies

Cells were loaded with 6-methoxy-N (3 sulfopropyl) quinolinium (SPQ) (from Molecular Probes, Europe, BV, Leiden, Netherlands) by exposure to a low osmolality external medium (150 mosmoles/l) containing 7.5 mmol/l SPQ for 4 min, as proposed by Chao et al. (1989) and Verkman et al. (1989). When microspectrofluorimetry was combined with electrophysiology as described earlier (Garcia et al. 1997a,b) the whole-cell mode of the patch-clamp technique was used (Hamill et al. 1981) and pipette media was supplemented with 0.5 mmol/l SPQ, in order to compensate for cellular probe dialysis. This concentration statistically equilibrated cell SPQ concentration. For combined experiments, the extracellular medium (EM) contained (in mmol/l): 137 NaCl, 5.4 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 0.4 or 10 $NaHCO_3$, 0.3 Na_2 HPO_4, 0.4 KH_2 PO_4, 10 HEPES, and 10 glucose. In some experiments NaCl was replaced equimolarly by Na-methane sulfonate, $CaCl_2$ by Ca-gluconate and $MgCl_2$ by $MgSO_4$ to expose the cells to deprived-chloride medium. The intra-pipette medium was formulated with 140 mmol/l potassium gluconate (Kgluc), 2 mmol/l $MgCl_2$, 5 mmol/l 1.1, N-2-hydroxyethyl piperazine-N4-2-ethane sulfonic acid (HEPES), 1.1 mmol/l ethylene glycol-bis (beta-amino ethylether), and N,N,N′,N′-tetraacetic acid (EGTA), giving a reversal potential of ~ -90 mV for Cl^- ($Erev_{Cl^-}$). As SPQ is a single-excitation, simple-emission, Cl^--sensitive molecule, relative variations in fluorescence are usually considered to demonstrate changes in Cl^- concentrations (Verkman et al. 1989). In these conditions we assumed that the initial rise in SPQ fluorescence (F_{SPQ}), observed after the patch rupture, was due to Cl^- dialysis toward the pipette where the Cl^- concentration is 4 mmol/l (Kgluc). Other possible sources of interferences independent of $[Cl^-]_i$, such as cell volume or pH_i changes were shown to be of little importance in F_{SPQ} variations (Foskett 1990; Sartor et al. 2004). F_{SPQ} was monitored using an inverted microscope (Nikon, Paris, France) equipped for microspectrofluorimetry (for more details, see Sartor et al. 2004).

pH_i studies

The fluorescent probe seminaphtorodafluor (SNARF-1) (Molecular Probes Europe, Leiden, Netherlands) was used to measure pH_i. Cells on their coverslips were incubated at 35 ± 1 ℃ for 30 min with the permeant acetoxymethyl ester (AM) form, then rinsed and transferred for examination with the epifluorescence microscope. The excitation wavelength was set at 514 nm (10 nm bandwidth) and emission centered at 580 nm for the acidic form of the probe and 640 nm for the alkaline form. The F580/640 ratio was recorded on line by an analog divider and used to calculate the pH_i of individual cells. Ratio traces were obtained on a chart recorder (Mariot et al. 199l).

Cell energetic studies

Respiratory chain activity

Cells grown on collagen beads were used on the 5^{th} day, 24 h after the last medium change. Fractions of the control and of the harvested cells culture media were frozen for glucose and lactate dosages. Cell culture density was estimated after digestion of the beads by collagenase. Immunocytochemical determination of lactotrope cells proportion was realized on the same population.

After rinsing with saline solutions containing either 140 or 0 mmol/l Cl^-, 0.2 to 1×10^6 cells on their collagen beads were introduced into two 2 ml cuves of an Oroboros oxygraph (Paar, Graz, Austria). Oxygen consumption was measured with a Clark electrode at 28 ℃ under permanent and regular agitation.

Glucose consumption

In order to determine glucose consumption and glucose metabolic pathways during the 24 h preceding respiration measurements, the concentration of glucose in the culture medium was determined using an automatic analyzer with glucose oxydase and peroxydase for lactate production.

ATP and ADP concentration measurements were carried out on 10 % of the cells processed for respiration experiments. After total collagenase digestion of the beads, the cells were lyzed under the action of 50 μl of 65 % perchloric acid (containing 50 mmol/l EDTA) for 300–400 μl of supernatant, centrifuged at $3500 \times g$ for 5 min, the resulting supernatant separated and its pH adjusted to 6.2–6.8 with a solution of KOH (2N) mixed with morpholinopropane-sulfonic acid (MOPS 0.3 mol/l), and frozen until assay. ATP concentration was determined using the enzymatic reaction luciferine-luciferase (Lundin et al. 1976). Bioluminescence was measured with a bioluminometer. The final concentration was estimated by comparison with a control assay (known concentrations of ATP). ADP was measured after transformation into ATP by pyruvate kinase in the presence of phosphoenol pyruvate (PEP). To this amount (ATP + ADP) we subtracted the ATP quantity, measured in the presence of PEP only, and obtained thus the ADP concentration. ATP/ADP ratio was then calculated.

Results

Interactions between extracellular Cl^- concentrations, $[Cl^-]_i$ and cellular energy

F_{SPQ} kinetics of cells incubated in Cl^--deprived medium was compared to control cells bathed in normal Cl^- medium (140 mmol/l), during patch clamp recordings with pipettes containing 4 mmol/l of Cl^-. At the patch rupture, F_{SPQ} increased in control cells, whereas it decreases in absence of extracellular Cl^-, indicating a Cl^- flux from the pipette to the cytosol (Fig. 1A). This means that $[Cl^-]_i$ is below 4 mmol/l and close to 0 mmol/l in this experimental situation.

Intracellular content of ATP was decreased after short incubation periods (1 min) in Cl^--deprived medium (82.2 ± 2.7 %, compared to 100 % for normalized

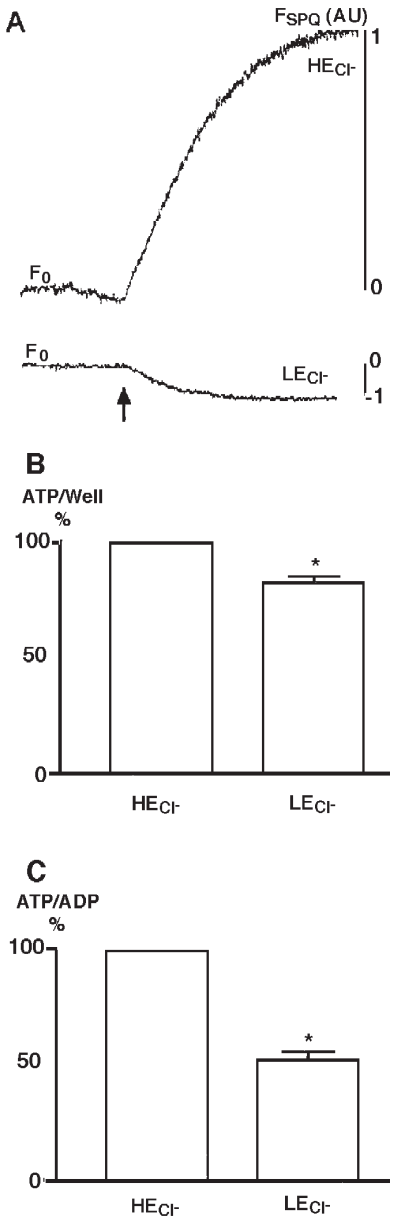

Figure 1. Extracellular Cl^- concentration influences intracellular Cl^- concentration ($[Cl^-]_i$) and cell ATP contents. **A**. Effect of extracellular Cl^- changes on $[Cl^-]_i$ revealed by F_{SPQ} recordings. Lactotrope cells were loaded with the chloride sensitive probe SPQ and patched with an intra-pipette medium containing 4 mmol/l Cl^- (Kgluc). Shortly after the patch rupture (F_0), they decreased their $[Cl^-]_i$ down to 4 mmol/l (upper trace, F_{SPQ} increase) in presence of 150 mmol/l Cl^- (high extracellular Cl^-, HE_{Cl-}) in the external medium (EM). In contrast, they increased (F_{SPQ} decrease) their $[Cl^-]_i$ up to the pipette concentration in presence of a Cl^- free EM (low extracellular Cl^-, LE_{Cl-} lower trace). This means that Cl^--free EM imposes to the cell a $[Cl^-]_i$ close to 0 mmol/l. AU: arbitrary units. **B**. Cell ATP concentration *per* well is decreased by short incubations (1 min, * $p < 5\%$) in Cl^--free EM. **C**. ATP/ADP ratio is also affected by Cl^--free EM (* $p < 5\%$).

controls, Fig. 1B). ATP concentration was around 600 pmoles *per* well. After ADP concentration measurements, ATP/ADP ratio was calculated and we observed a very large decrease ($52.7 \pm 3.5\%$, compared to 100% for normalized controls, Fig. 1C) under the Cl^--deprived culture conditions. When pH_i was measured for

cells bathing in normal or Cl^--deprived medium, we found a shift from 7.34 ± 0.01 ($n = 132$ cells) to 7.84 ± 0.01 ($n = 50$ cells). It appears that more information is needed about the interplay between $[Cl^-]_i$, respiratory chain activity and pH_i.

When a mitochondrial uncoupler such as carbonyl cyanide m-phenylhydrazone (50 μmol/l) was added to normal Cl^- medium, ATP content of cells grown in 24 wells plates was decreased after 1 min at $80.3 \pm 2.5\,\%$ (7 experiments with 3 assays in duplicate for each experiment) of the control cells. This value is close to that observed with Cl^--deprived medium (Fig. 1B). It was then of interest to study the consequence of $[Cl^-]$i decrease on the respiratory chain activity.

$[Cl^-]_i$ influences respiratory chain function

Pituitary cells grown on collagen beads were introduced into 2 ml cuvettes to monitor oxygen consumption in normal and Cl^--deprived medium, with Clark electrode sensitive to oxygen. We found that low $[Cl^-]_i$ resulting from the absence of extracellular Cl^-, induced a significant increase in the respiratory chain activity for low respiratory rates (Fig. 2A). However, when higher respiratory rates (>2.5 nat $O_2/10^6$ cells/min) were observed under control conditions, the effect of Cl^--deprived medium was inversed. We observed a decrease in oxygen consumption (Fig. 2B). At the end of the assay, the oxygen concentration decrease independent of respiration was estimated under potassium cyanide (KCN). The results relative to real cellular oxygen consumption are summarized in Fig. 2C,D (1.67 ± 0.16 *vs.* 2.41 ± 0.26 nat $O_2/10^6$ cells/min for cells exhibiting a low respiratory rate (LRR) incubated into high or low chloride EM, respectively, and 5.19 ± 0.84 *vs.* 2.83 ± 0.76 nat $O_2/10^6$ cells/min for cells exhibiting a high respiratory rate (HRR) incubated into high or low chloride EM, respectively ($n = 10$ in each case, Fig. 2D). We thus observed two different cell populations as a function of their respective respiratory performances ($p < 1\,\%$ for LRR and HRR cell populations). The data also established that these cell populations responded in an opposite manner to chloride depletion as concern their respiratory chain activity (an increase for LRR and a decrease for HRR cells). In both cases the effect was significant ($p < 5\,\%$). This suggests that a neutral state is obtained in the absence of the anion.

To investigate more finely the metabolic pathways underlying the energetic processes in lactotrope cells, we analyzed the relations between respiratory chain activity and glucose consumption.

Correlation with glucose consumption

In order to determine the pathway that was preferentially used by the cell to generate ATP, we analyzed the fate of medium glucose. Extracellular glucose concentration was measured at the beginning and at the end of the last 24 h period of culture, glucose consumption was correlated to the release of lactic acid in the culture medium for the same period and the respiration rate was measured. The ratio lactate formed/glucose used gives information on the relative importance of anaer-

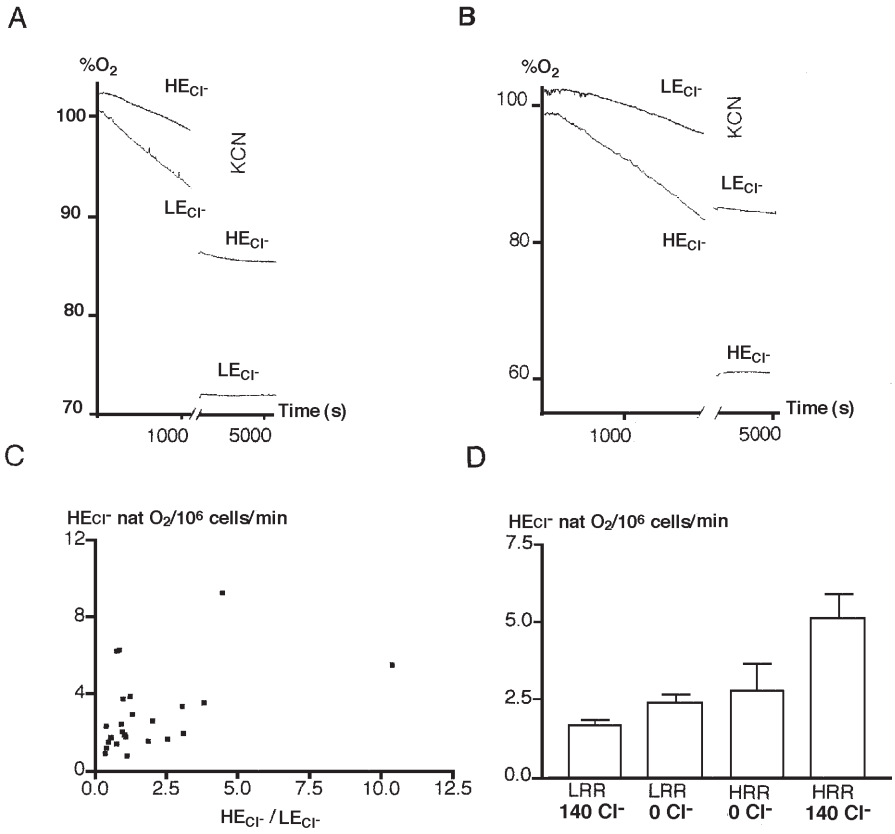

Figure 2. Respiratory performance in presence of either high or low external chloride. **A**. Cell characterized by a low respiratory rate (LRR) in presence of HE_{Cl-}, shows an increased oxygen consumption under LE_{Cl-}. Effect of potassium cyanide (KCN, 1 mmol/l) on respiratory performance at the end of the recording. **B**. Cell exhibiting a high respiratory rate (HRR) in presence of HE_{Cl-} shows a decreased oxygen consumption under LE_{Cl-}. Effect of KCN (1 mmol/l) on respiratory performance at the end of the recording. **C**. Relation between HE_{Cl-} oxygen consumption (nat/10^6 cells/min) and HE_{Cl-}/LE_{Cl-} oxygen consumption ratios. **D**. Comparison of Cl^--deprived medium effects on LRR and HRR cells ($p < 1\%$, Mann–Whitney nonparametric test).

obic and aerobic glycolysis and on the possible participation of other metabolic pathways to lactate formation (see Discussion).

ATP and ADP concentrations were determined on cells aliquots sampled at the beginning of the respiration experiments. When ATP/ADP and lactate/glucose (L/G) ratios were compared to the respiratory performance, we found that ATP/ADP and L/G ratios were inversely correlated to the oxygen consumption (Fig. 3A). The higher respiratory rate, the lower were the ATP/ADP and L/G ratios. This was

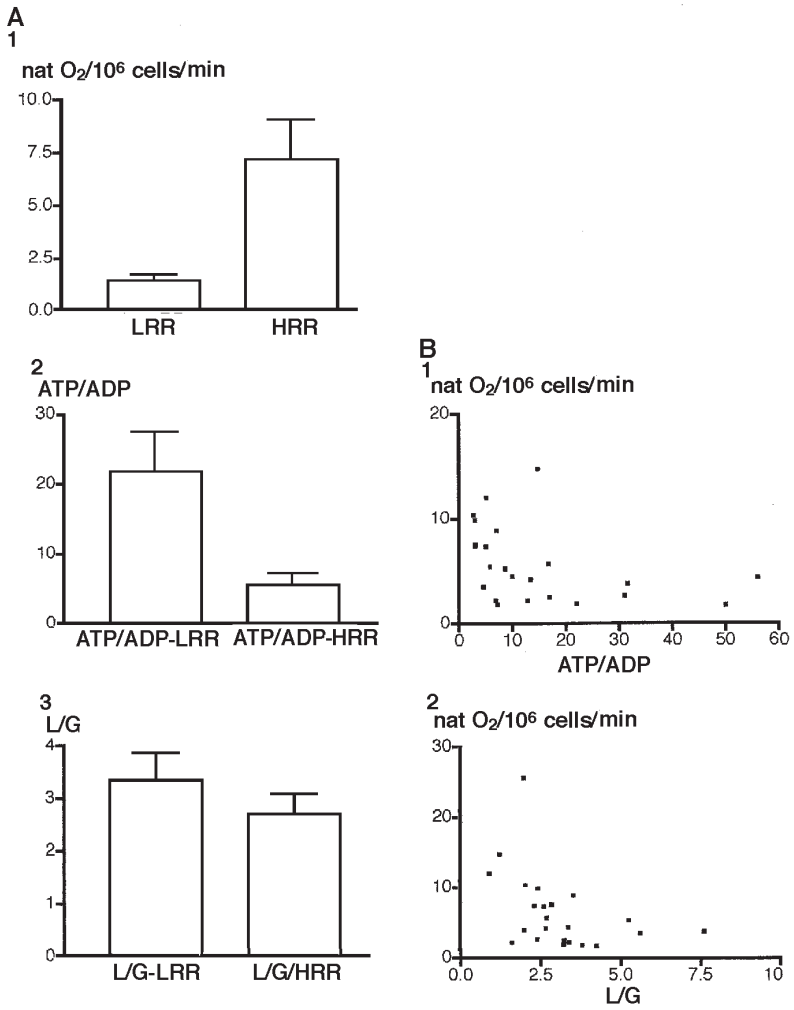

Figure 3. Correlation between respiration and metabolic parameters. **A**. Comparison of synthesized lactate/used glucose (L/G) and ATP/ADP ratios as a function of the respiratory rate. 1. LRR and HRR comparisons. The populations under and above 2.5 nat $O_2/10^6$ cells/min have been separated. These values represent the mean respiration rate of populations studied in A2, A3, B1, B2. 2. ATP/ADP ratios comparison. 3. L/G ratios comparisons. **B.** Correlation between respiratory rates and: 1. ATP/ADP, 2. L/G.

verified statistically, ($p < 5\%$), for both ratios when we compared rough respirations results (not shown) but only ATP/ADP were inversely correlated for results based on KCN sensitive oxygen consumption. A non-linear correlation between these parameters is suggested by the data shown in Fig. 3B.

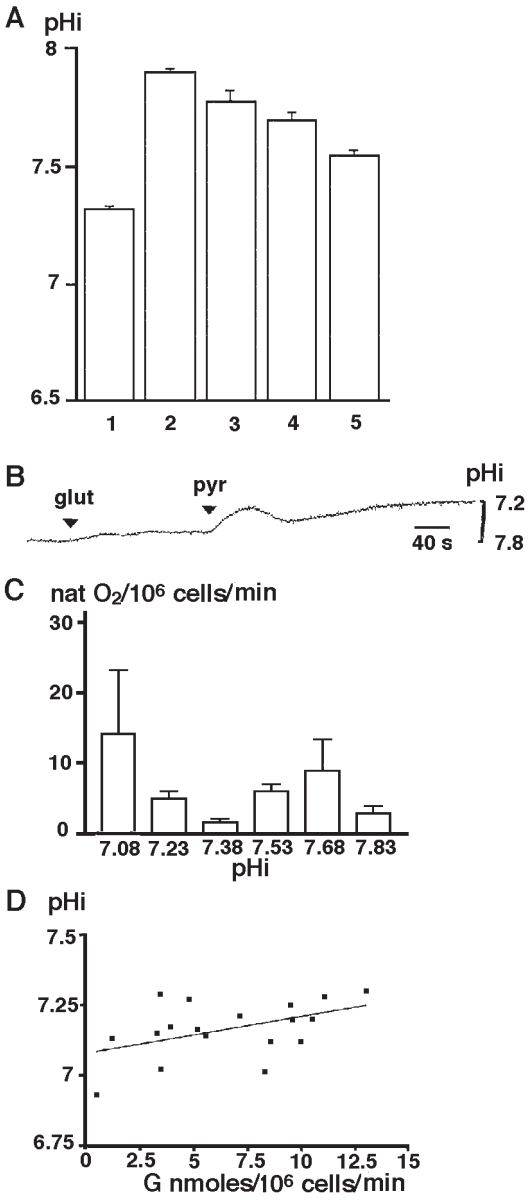

Figure 4. Interactions between pH_i, metabolic and respiratory parameters. **A.** pH_i is influenced by metabolites such as glutamine (glut) and/or pyruvate (pyr). SNARF-1 loaded cells pH_i were recorded in control and alkalized cells (HE_{Cl-} replaced by LE_{Cl-} + 10 mmol/l bicarbonate, columns 1 and 2, respectively). Glut and pyr (columns 3 and 4, respectively) were able to acidify the cytosol. Their effects are additive (column 5). **B**. Individual recording of pH_i showing the acid loading induced by pyr and glut. **C**. Correlation between pH_i and respiration (rough values were used in this figure because KCN effects were not determined for all respiration assays). Variations of 0.15 unity of pH were used to analyse the populations. Populations of alkalized cells were added to control populations. Cells with a pH_i between 7.3 and 7.44 have lower respiratory rates, significantly different from the others ($p < 5\%$), except for the most alkalized population (7.75–7.89). **D**. Correlation between pH_i and glucose (G) consumption. $r = 0.47$, $p < 5\%$.

The second important observation deals with the fact that L/G ratios were barely under two (5/24). This implies that: (i) aerobic glycolysis is poorly used compared to the anaerobic processes and (ii) glycolysis is not the only biochemical pathway providing pyruvate to the cell machinery. One of the putative pathways

probably uses glutamine. The addition of pyruvate in the culture medium is also to be considered as a possible source for lactate production, especially when oxygen fluxes are low.

Interaction with pH_i

In fact, pyruvate and (or) glutamine were able to modulate pH_i. The incubation of lactotrope cells into Cl^--deprived medium led to an alkalinization that reversed under pyruvate and (or) glutamine (Fig. 4A,B). We thus investigated interactions between pH_i and cell respiration in lactotrope cells grown simultaneously on collagen beads and on glass coverslips. Respiration and pH_i data obtained with cells tested under normal or Cl^--deprived medium were compared. LRR were correlated with pH_i around 7.3–7.4, whereas more acidic or alkalinized pH_i were associated with HRR (Fig. 4C).

When glucose consumption rates were correlated with pH_i, we found that increased pH_i of cells cultured in normal Cl^- medium were associated with increased glucose consumption (Fig. 4D). Lactate formation was also enhanced (not shown).

Discussion

The aim of this study was to investigate the relationships between Cl^-, energy metabolism and pH_i in rat lactotrope cells. We provide evidence that Cl^- interferes with cell energy metabolism. Low $[Cl^-]_i$ decrease ATP/ADP ratio and affects respiratory chain activity. However, the aerobic pathway for glucose metabolism seems to be less important than the anaerobic one giving lactate. Finally, pH_i around 7.3–7.4, which is correlated with the lower respiratory chain activity is likely the best pH_i level to insure the higher ATP/ADP ratio to the cell by promoting the anaerobic pathway.

An indirect demonstration that $[Cl^-]_i$ could modify intracellular ATP concentration ($[ATP]_i$) was provided by DeWeille and Lazdunski (1990), when they demonstrated that decreased $[Cl^-]_i$ affected the activity of the ATP-dependent K^+ channel of insulinoma cells. Our results are in agreement with these data and allow us to propose a mechanism of interaction between $[Cl^-]_i$ and $[ATP]_i$. The presence of Cl^- stores in mitochondria of rat lactotrope cells (Garcia et al. 1997b), can be linked to the observed effects of $[Cl^-]_i$ changes on cell respiration performance, whatever may be the case (LRR or HRR).

Our observation of similar respiratory performances of cells incubated in chloride-deprived medium, independently of their respiratory rate (HRR or LRR) in normal chloride medium, means that $[Cl^-]_i$ is not the only parameter involved in cell energetic status. It also strengthens the idea that lactotrope cells may present several physiological status, (Sartor et al. 2004) linked to complex interactions between $[Cl^-]_i$, pH_i, and cell energetics. These results are in agreement with earlier reports by Zhang et al. (1990), Lledo et al. (1991) who described two populations in rat lactotrophs cells. Finally the fact that LRR were associated with high ATP/ADP

ATP/ADP ratio is in favor of a thermodynamic control of respiratory chain activity by the phosphate potential (ATP/ADP, inorganic phosphate).

Over the last decades, increasing evidence has been presented, indicating that glucose was not the main fuel for cell energy supply in a number of cell types including tumoral cells (Baggetto 1992; Martin et al. 1998), neoplastic or normal cells (Board et al. 1990), cultured mammalian cells (Zielke et al. 1984) and that, in cultured mammalian cell lines, glucose was metabolized *via* glycolysis and lactate formation (Neermann and Wagner 1996). Rappay et al. (1979) demonstrated that rat anterior pituitary cells in culture used the anaerobic glycolysis to insure their energy metabolism. The L/G ratios reported in this study are in full agreement with these previous reports. We can postulate that most of the glucose used is metabolized *via* the anaerobic pathway. In addition, L/G ratios above 2 are explained by the occurrence of one or more additional metabolic pathways. Vannucci and Hawkins (1983) for their part concluded that glucose was not the main fuel for pituitary cell energy supply. In fact, glutamine has been proposed as a major energy source for mammalian cells in culture (Zielke et al. 1984). However, this does not rule out a possible intervention of other metabolic pathways (e.g fatty acids). Taken together, lactic fermentation appears as the main cellular mechanism used to insure energetic supply.

To understand the balance between the two states of HRR and LRR, we examined the relation of these parameters with pH_i in normal and chloride-deprived media. Our interpretation, based on data from Board et al. (1990) on the activities of glycolysis and tricarboxylic acid cycle key enzymes, is that the tricarboxylic acid cycle might be shunted. When the pH_i is around 7.4, it could activate the pyruvate-malate shuttle and thus decrease the tricarboxylic acid cycle and respiratory chain activities to the benefit of the lactate pathway. The pH_i values, found in our experiments, are in agreement with those reported by Board et al. (1990) for these two pathways.

The presence of an anionic exchange system ($Cl^-/^-HCO_3$) involved in pH_i and $[Cl^-]_i$ regulations which are energy dependent, as reported in previous papers (Garcia et al. 1997c; Sartor et al. 2004), make this proposal coherent. Complex interactions, based on multiple regulatory sites rather than a single enzyme activity change, have been proposed for metabolic oscillations in pancreatic β-cells (McDonald et al. 1997). Thus, this kind of regulation may also help to explain the phenotypic differences observed in lactotropes cells.

We can speculate on the physiological significance of such mechanisms. Cherubini and Ben-Ari (1989), Krnjevic et al. (1989), Ben-Ari (1992) observed that immature neurons resist more efficiently to hypoxia than mature ones. Data from Longuemare et al. (1994), indicated that hypoxia alone was not able to decrease ATP levels and to increase the astrocyte extracellular accumulation of excitatory acids, which are partly responsible for ischemic neuronal injury. The main source of cellular ATP has thus a clear anaerobic origin. We know that Cl^- regulation mechanisms such as chloride cotransport systems, play a primordial role during neuronal maturation (Rivera et al. 1999). We have also demonstrated the role of

chloride in pH_i regulation system (Garcia et al. 1997c) and its interaction with mitochondria function (Garcia et al. 1997b). Our present results are in favor of a pivotal role for $[Cl^-]_i$ variations on cellular energetic reserves. We may hypothesize that a balance between anaerobic and aerobic pathways explains the different effect of oxygen deprivation in immature and mature neurons. Tolerance of cerebral anoxia in turtles cortical brain slices, related to anaerobic ATP production (Bickler 1992), might be explained by similar mechanisms.

Finally, we suggest that the role played by chloride in the balance between aerobic and anaerobic pathways, for cell energy production in lactotrope cells, might be present in other cell types, especially in neurons during the immaturity-maturity transition, and explain some phenotypic alterations of the genotype.

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