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# $Ca^{2+}$ dependence of gluconeogenesis stimulation by glucagon at different cytosolic $NAD^+$-NADH redox potentials

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## Abstract

The influence of $Ca^{2+}$ on hepatic gluconeogenesis was measured in the isolated perfused rat liver at different cytosolic $NAD^+$-NADH potentials. Lactate and pyruvate were the gluconeogenic substrates and the cytosolic $NAD^+$-NADH potentials were changed by varying the lactate to pyruvate ratios from 0.01 to 100. The following results were obtained: a) gluconeogenesis from lactate plus pyruvate was not affected by $Ca^{2+}$-free perfusion (no $Ca^{2+}$ in the perfusion fluid combined with previous depletion of the intracellular pools); gluconeogenesis was also poorly dependent on the lactate to pyruvate ratios in the range of 0.1 to 100; only for a ratio equal to 0.01 was a significantly smaller gluconeogenic activity observed in comparison to the other ratios. b) In the presence of $Ca^{2+}$, the increase in oxygen uptake caused by the infusion of lactate plus pyruvate at a ratio equal to 10 was the most pronounced one; in $Ca^{2+}$-free perfusion the increase in oxygen uptake caused by lactate plus pyruvate infusion tended to be higher for all lactate to pyruvate ratios; the most pronounced difference was observed for a lactate/pyruvate ratio equal to 1. c) In the presence of $Ca^{2+}$ the effects of glucagon on gluconeogenesis showed a positive correlation with the lactate to pyruvate ratios; for a ratio equal to 0.01 no stimulation occurred, but in the 0.1 to 100 range stimulation increased progressively, producing a clear parabolic dependence between the effects of glucagon and the lactate to pyruvate ratio. d) In the absence of $Ca^{2+}$ the relationship between the changes caused by glucagon in gluconeogenesis and the lactate to pyruvate ratio was substantially changed; the dependence curve was no longer parabolic but sigmoidal in shape with a plateau beginning at a lactate/pyruvate ratio equal to 1; there was inhibition at the lactate to pyruvate ratios of 0.01 and 0.1 and a constant stimulation starting with a ratio equal to 1; for the lactate to pyruvate ratios of 10 and 100, stimulation caused by glucagon was much smaller than that found when $Ca^{2+}$ was present. e) The effects of glucagon on oxygen uptake in the presence of $Ca^{2+}$ showed a parabolic relationship with the lactate to pyruvate ratios which was closely similar to that found in the case of gluconeogenesis;

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the only difference was that inhibition rather than stimulation of oxygen uptake was observed for a lactate to pyruvate ratio equal to 0.01; progressive stimulation was observed in the 0.1 to 100 range. f) In the absence of $Ca^{2+}$ the effects of glucagon on oxygen uptake were different; the dependence curve was sigmoidal at the onset, with a well-defined maximum at a lactate to pyruvate ratio equal to 1; this maximum was followed by a steady decline at higher ratios; at the ratios of 0.01 and 0.1 inhibition took place; oxygen uptake stimulation caused by glucagon was generally lower in the absence of $Ca^{2+}$ except when the lactate to pyruvate ratio was equal to 1. The results of the present study demonstrate that stimulation of gluconeogenesis by glucagon depends on $Ca^{2+}$. However, $Ca^{2+}$ is only effective in helping gluconeogenesis stimulation by glucagon at highly negative redox potentials of the cytosolic $NAD^+$-NADH system. The triple interdependence of glucagon-$Ca^{2+}$-$NAD^+$-NADH redox potential reveals highly complex interrelations that can only be partially understood at the present stage of knowledge.

## Introduction

Although primarily mediated by cAMP, the action of glucagon on hepatic metabolism can be influenced by several factors. In the specific case of gluconeogenesis there are two factors that seem to be of striking importance: a) the intracellular concentration and distribution of $Ca^{2+}$ (1) and b) the redox state of the $NAD^+$-NADH couple (2).

Friedmann and Park (3) showed that glucagon administration to the perfused liver causes the release of previously accumulated $Ca^{2+}$. Subsequently, it was demonstrated that glucagon, as well as ß-adrenergic agents, are able to induce $Ca^{2+}$ influx (4-8). As a consequence of these $Ca^{2+}$ movements, i.e., influx and release from the intracellular pools, administration of glucagon to the liver cells is followed by an increase in the cytosolic $Ca^{2+}$ concentration (9,10). Since every hormone that increases gluconeogenesis evokes changes in $Ca^{2+}$ fluxes it is generally believed that the action of glucagon on gluconeogenesis is also dependent, at least to some extent, on $Ca^{2+}$. In fact, it was demonstrated that several gluconeogenic key enzymes such as pyruvate carboxylase (11) and protein kinases (12) are stimulated by $Ca^{2+}$. Additionally, gluconeogenesis is an energy-dependent process and several respiratory enzymes are sensitive to $Ca^{2+}$ (13). It was indeed demonstrated in the perfused liver that the effects of suboptimal glucagon concentrations on oxygen consumption were more pronounced when $Ca^{2+}$ was present as opposed to the condition in which $Ca^{2+}$ was omitted from the perfusion fluid and the intracellular pools had been exhausted (14).

The influence of the cytosolic $NAD^+$-NADH redox potential on the effects of glucagon on gluconeogenesis has been investigated mainly by changing the lactate to pyruvate ratios. Besides being gluconeogenic substrates, the compounds are rapidly interconverted by the near equilibrium enzyme lactate dehydrogenase so that changes in the lactate to pyruvate ratio also produce changes in the cytosolic $NAD^+$-NADH redox potential (15). By using this approach, several investigators have shown that the stimulating action of glucagon on gluconeogenesis is more pronounced at low (more negative or reduced) $NAD^+$-NADH potentials (2,16,17). At high potentials the stimulating action of glucagon may be either abolished or transformed into inhibition.

Rashed and Patel (18), on the other hand, have demonstrated that a link between $Ca^{2+}$ movements induced by glucagon and the cellular redox potential is likely to exist. These investigators found that the glucagon-stimulated $Ca^{2+}$ efflux in the isolated perfused rat liver is dependent on the cellular redox potential. Furthermore, it is well known that both the intracellular $Ca^{2+}$ concentra-

tion/distribution and the redox potential of the $NAD^+$-NADH couple are parameters which have in common the ability of influencing the rate of mitochondrial respiration. Gluconeogenesis is strictly dependent on ATP generated within the mitochondria and, for this reason, the increases in gluconeogenesis caused by glucagon are always coupled to increases in oxygen uptake (19). Therefore, one should expect reciprocal influences of $Ca^{2+}$ and the $NAD^+$-NADH redox potential on gluconeogenesis stimulation by glucagon. Exactly how these two parameters affect the action of glucagon, however, has not been investigated and the present study represents an attempt to clarify this question. The experimental system was the isolated rat liver perfused with Krebs/Henseleit-bicarbonate buffer. Gluconeogenesis was measured by simultaneously varying the cytosolic $NAD^+$-NADH redox potential and the cellular $Ca^{2+}$ content.

## Material and Methods

### Liver perfusion

Male albino Wistar rats weighing 180-220 g and fed a standard laboratory diet (Purina®, São Paulo, Brazil) *ad libitum* were used. Food was withdrawn 24 h prior to the liver perfusion experiments. For the surgical procedure the rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). Hemoglobin-free, non-recirculating perfusion was performed. The surgical technique of Scholz and Bücher (20) was used. After cannulation of the portal vein and the vena cava the liver was positioned in a plexiglass chamber. The flow was maintained constant with a peristaltic pump. The perfusion fluid was Krebs/Henseleit-bicarbonate buffer, pH 7.4, saturated with a mixture of oxygen and carbon dioxide (95:5) by means of a membrane oxygenator with simultaneous temperature adjustment at 37°C.

### $Ca^{2+}$-free perfusion

For $Ca^{2+}$-free perfusion, the intracellular $Ca^{2+}$ pools were depleted. Livers were preperfused with $Ca^{2+}$-free Krebs/Henseleit-bicarbonate buffer containing 0.2 mM ethylenediamine tetraacetate (EDTA), 5 mM glucose, 1 mM lactate and 0.1 mM pyruvate. In order to ensure maximal depletion of the intracellular $Ca^{2+}$ pools, phenylephrine (2 µM) was infused repeatedly (3 times) for short periods of time (2 min) at 5-min intervals. According to Reinhart et al. (21), this procedure depletes the intracellular $Ca^{2+}$ pools which are normally mobilized when hormones are infused.

### Enzymatic assays

Samples of the effluent perfusion fluid were collected according to the experimental protocol and analyzed for metabolite content. Glucose (22), L-lactate (23) and pyruvate (24) were assayed by standard enzymatic procedures. The oxygen concentration in the outflowing perfusate was monitored continuously using a teflon-shielded platinum electrode adequately positioned in a plexiglass chamber at the exit of the perfusate (25). Metabolic rates were calculated from input-output differences and the total flow rates and were referred to the wet weight of the liver.

### Material

The liver perfusion apparatus was built in the workshops of the University of Maringá. All enzymes and coenzymes used in the enzymatic assays were purchased from Sigma Chemical Co. (St. Louis, MO). Crystalline glucagon was purchased from Eli Lilly do Brasil Ltda., diluted in Krebs/Henseleit-bicarbonate buffer and stored at 4°C. All chemicals were of the best available grade.

### Statistical analysis

The statistical significance of the differ-

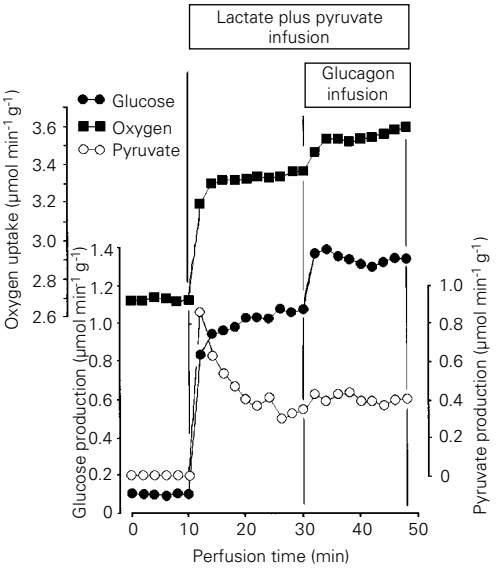


Figure 1 - Time course of the effects of glucagon on glucose production, oxygen uptake and pyruvate production. Livers from fasted rats were perfused with Krebs/Henseleit-bicarbonate buffer containing 2.5 mM $CaCl_2$, as described in Material and Methods. Lactate plus pyruvate (3.4 and 0.35 mM, respectively) and glucagon (10 nM) were infused as indicated by the horizontal bars. Samples of the outflowing perfusate were taken at 2-min intervals and glucose and pyruvate were measured enzymatically. Oxygen uptake was followed polarographically. Each data point represents the mean of 3 liver perfusion experiments.

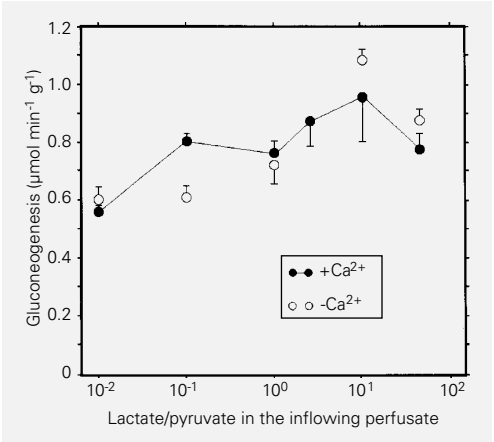


Figure 2 - Gluconeogenesis in the rat liver as a function of the lactate to pyruvate ratio in the presence (filled circles) and absence (open circles) of $Ca^{2+}$. Livers from fasted rats were perfused as described in Material and Methods. The experiments were performed according to the experimental protocol illustrated in Figure 1. The control experiments (filled circles) were performed with Krebs/Henseleit-bicarbonate buffer containing 2.5 mM $CaCl_2$. In the $Ca^{2+}$-free experiments (open circles), $CaCl_2$ was omitted from the perfusion fluid and the intracellular $Ca^{2+}$ pools were previously exhausted by repeated phenylephrine infusion in the presence of 0.2 mM EDTA. Initially substrate-free perfusion was performed. The infusion of lactate and pyruvate was initiated after oxygen uptake stabilization. The lactate to pyruvate ratio was changed from 0.01 to 100, with a total lactate plus pyruvate concentration of 3.75 mM. Gluconeogenesis represents the final steady-state rate of glucose release by the liver after 20 min of lactate + pyruvate infusion. Each data point represents the mean of 3 to 5 liver perfusion experiments. The vertical bars represent the standard errors of the mean.

ences between parameters was evaluated with the Primer program, version 1.0 (MacGraw-Hill). The Student *t*-test, the paired Student *t*-test and the Student-Newman-Keuls test were applied for data analysis, with the level of significance set at $P<0.05$.

## Results

### Experimental protocol

The experimental protocol used in the present study is illustrated in Figure 1. Livers from 24-h fasted rats were perfused with complete Krebs/Henseleit-bicarbonate buffer (2.5 mM $CaCl_2$). After oxygen uptake stabilization, sampling of the effluent perfusate was started and infusion of the gluconeogenic substrates was initiated 10 min later. The substrates were lactate and pyruvate infused at a total concentration of 3.75 mM. The lactate to pyruvate ratios, however, ranged from 0.01 to 100. On the basis of the lactate dehydrogenase equilibrium, changes of up to 280 mV in the cytosolic $NAD^+$-NADH potential are expected (15). In the experiment shown in Figure 1, the lactate to pyruvate ratio was equal to 10. The basal rates of glucose production were very low. Upon infusion of lactate plus pyruvate, glucose production increased considerably and reached a new steady state within the next 20 min. The introduction of glucagon increased glucose production to a new steady state. Oxygen uptake followed more or less the same pattern. Pyruvate production showed a maximum immediately after the beginning of infusion, but at later times it also tended to be stable.

### Effect of $Ca^{2+}$ on gluconeogenesis at different lactate to pyruvate ratios

The behavior illustrated in Figure 1 can be analyzed in terms of steady-state rates and/or differences between steady states. The first question that arises is about the rates of

glucose production in the presence and absence of $Ca^{2+}$ at different cytosolic $NAD^+$-NADH potentials. Figure 2 provides an answer to this question. In this figure the steady-state rates of glucose production are plotted against the lactate to pyruvate ratio. Two series of experiments were performed, i.e., control experiments and the $Ca^{2+}$-free experiments. The latter were performed with $Ca^{2+}$-free Krebs/Henseleit-bicarbonate buffer after depletion of intracellular $Ca^{2+}$ by repeated phenylephrine infusions (see Material and Methods). As shown in Figure 2, gluconeogenesis presented relatively small changes in the lactate to pyruvate ratio. For lactate to pyruvate ratios of 0.1 to 100 gluconeogenesis was similar; at lactate/pyruvate ratios equal to 0.01, however, it was significantly smaller ($P<0.05$, Student-Newman-Keuls test). $Ca^{2+}$-free perfusion also had little influence on the rates of gluconeogenesis. The most pronounced difference was found for a lactate/pyruvate ratio equal to 0.1, with gluconeogenesis being 25% lower in the absence of $Ca^{2+}$. However, no systematic tendencies were apparent so that one may conclude that $Ca^{2+}$ is virtually without effect on hepatic basal gluconeogenesis, irrespective of the cytosolic redox potential.

### Effect of $Ca^{2+}$ on the increase in oxygen uptake at different lactate to pyruvate ratios

In the perfused liver the infusion of lactate plus pyruvate is always followed by increases in oxygen uptake, which are presumed to occur in order to meet the increased needs in ATP for gluconeogenesis. In Figure 3 these oxygen uptake changes are presented against the lactate to pyruvate ratio for both the control experiments and the $Ca^{2+}$-free experiments. In the presence of $Ca^{2+}$ the increases in oxygen uptake were similar for all lactate to pyruvate ratios except for the ratio equal to 10, which caused a significantly higher increase ($P<0.05$, Student-Newman-Keuls test). In the absence of $Ca^{2+}$ the changes in oxygen uptake were similar to those observed for the controls at low lactate to pyruvate ratios (up to 0.1). With a lactate/pyruvate ratio between 1 and 100, however, oxygen uptake tended to respond more intensely to substrate infusion although a clear difference was only apparent for a lactate/pyruvate ratio equal to 1.0. Figure 3 reveals a relatively complex pattern. However, it is apparent that $Ca^{2+}$ has no positive effect on oxygen uptake in the presence of gluconeogenic substrates alone.

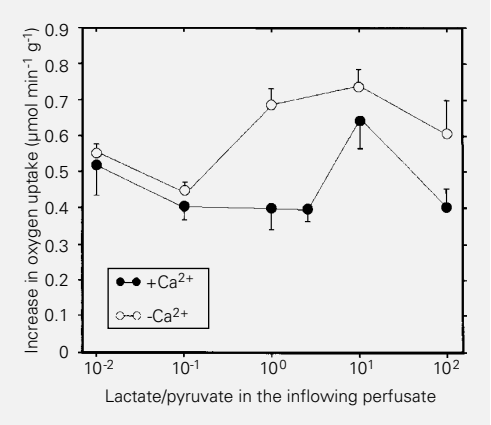


Figure 3 - Changes in oxygen consumption in the rat liver upon the infusion of lactate plus pyruvate at different ratios in the presence (filled circles) and absence (open circles) of $Ca^{2+}$. The data are from the same experiments as described in the legend to Figure 1. Oxygen uptake was followed polarographically. The changes are the differences between the new steady state in the presence of lactate plus pyruvate and the steady state during substrate-free perfusion. Each data point represents the mean of 3 to 5 liver perfusion experiments. The vertical bars represent the standard errors of the mean.

### Influence of the lactate to pyruvate ratio and $Ca^{2+}$ on the effects of glucagon on gluconeogenesis

Basal gluconeogenesis was barely affected by the lactate to pyruvate ratio, as shown above (Figure 2). The changes produced by glucagon, however, were closely correlated with the lactate to pyruvate ratio, as shown in Figure 4. In the presence of $Ca^{2+}$, glucagon had no effect at low lactate to pyruvate ratios. As this ratio was increased, however, the effects of glucagon increased progressively. Actually the relationship was clearly parabolic and it was indeed possible to fit a parabola to the experimental data, as described in the legend to Figure 4. The correlation coefficient was 0.987. In the ab-

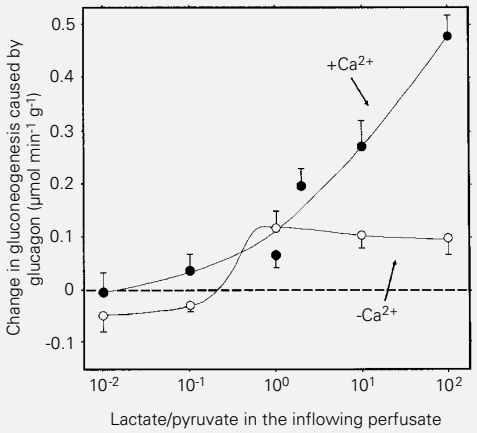


Figure 4 - Changes in gluconeogenesis caused by glucagon in the presence (filled circles) and absence (open circles) of $Ca^{2+}$ and at different cytosolic $NAD^+$-NADH redox potentials. Livers from fasted rats were perfused as described in Material and Methods and as illustrated in Figure 1. The changes in gluconeogenesis caused by glucagon are the differences between the new steady state in the presence of glucagon and the steady state in the absence of glucagon. Each data point represents the mean of 3 to 5 liver perfusion experiments. The vertical bars represent the standard errors of the mean. For the control curve (filled circles), the traced curve corresponds to a parabolic regression line, optimized by means of a least-squares procedure. The regression line was $\hat{y} = 0.0245 - 0.0637x + 0.031x^2$, with x = log (lactate/pyruvate x $10^3$). The parabolic correlation coefficient was 0.987.

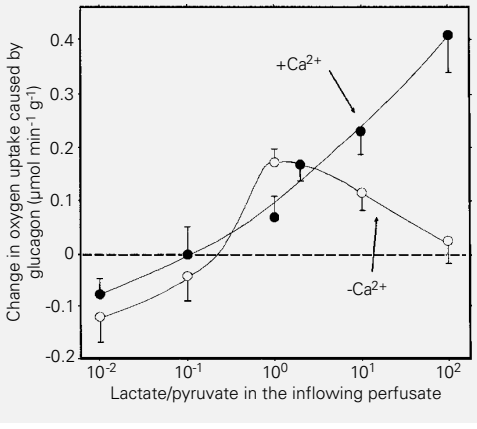


Figure 5 - Changes in oxygen uptake caused by glucagon in the presence (filled circles) and absence (open circles) of $Ca^{2+}$ and at different cytosolic $NAD^+$-NADH redox potentials. The data are from the same experiments as described in the legend to Figure 4. The changes in oxygen uptake caused by glucagon are the differences between the new steady state in the presence of glucagon and the steady state in the absence of glucagon. Each data point represents the mean of 3 to 5 liver perfusion experiments. The vertical bars represent the standard errors of the mean. For the control curve (filled circles), the traced line corresponds to a parabolic regression line, optimized by means of a least-squares procedure. The regression line was $\hat{y} = -0.116 + 0.0267x + 0.0156x^2$, with x = log (lactate/pyruvate x $10^3$). The parabolic correlation coefficient was 0.99.

sence of $Ca^{2+}$, on the other hand, the relationship between the effects of glucagon and the lactate to pyruvate ratios can be described as a sigmoidal one. Low inhibition was observed at low lactate/pyruvate values (0.01 and 0.1) and stimulation was observed beginning at a lactate/pyruvate ratio equal to 1.0. However, instead of increasing with the subsequent increases in lactate/pyruvate, the effects of glucagon reached a plateau. This plateau was well below the increases produced by glucagon at lactate/pyruvate values of 10 and 100 and in the presence of $Ca^{2+}$. Therefore, in the absence of $Ca^{2+}$ the effects of glucagon are different and much smaller at high lactate to pyruvate ratios.

### Influence of the lactate to pyruvate ratio and $Ca^{2+}$ on the effects of glucagon on oxygen uptake

The effects of glucagon on oxygen uptake at different lactate to pyruvate ratios are shown in Figure 5. In the presence of $Ca^{2+}$, the relationship between oxygen uptake increase and the lactate/pyruvate ratio was also clearly parabolic. Also in this case, a parabola, described in the legend to the figure, could be fitted to the experimental data, with a correlation coefficient of 0.99. Different from gluconeogenesis, however, the effect of glucagon at a lactate/pyruvate ratio equal to 0.01 was inhibitory even in the

presence of $Ca^{2+}$. This inhibition at low lactate/pyruvate values was enhanced in the absence of $Ca^{2+}$. The absence of $Ca^{2+}$ did not abolish oxygen uptake stimulation at a lactate/pyruvate ratio equal to 1.0. With further increases in the lactate/pyruvate ratio, however, stimulation of oxygen uptake decreased again in such a way that no significant stimulation was apparent at a lactate/pyruvate ratio equal to 100. Absence of $Ca^{2+}$, therefore, also changes the effects of glucagon on oxygen uptake in a way that depends on the cytosolic $NAD^{+}$-NADH redox potential.

### Influence of $Ca^{2+}$ on the effects of glucagon on lactate or pyruvate production

Under our experimental conditions, the liver presented net transformation of lactate into pyruvate at high lactate/pyruvate values; conversely, at low lactate/pyruvate values, a net transformation of pyruvate into lactate occurred. The former phenomenon represents net consumption of reducing equivalents by the liver, whereas the latter is a measure of the reducing equivalents that are lost by the liver. In livers from fasted rats these reducing equivalents are likely to come from the mitochondria and their production and transport out of these organelles depend on enzymes that are sensitive to both $Ca^{2+}$ and glucagon. In order to analyze this possibility, we evaluated the net lactate productions measured with inflowing lactate/pyruvate ratios equal to 0.01 and 0.1. Figure 6A shows the results obtained in the control and $Ca^{2+}$-free experiments. Lactate production was affected by both $Ca^{2+}$ and glucagon. When $Ca^{2+}$ was absent, lactate release was smaller and the effect was more pronounced when the inflowing lactate to pyruvate ratio was equal to 0.1. The introduction of glucagon decreased lactate production both in the presence and absence of $Ca^{2+}$.

Evaluation of the net rates of pyruvate production at lactate/pyruvate ratios equal to 10 and 100, as shown in Figure 6B, did not

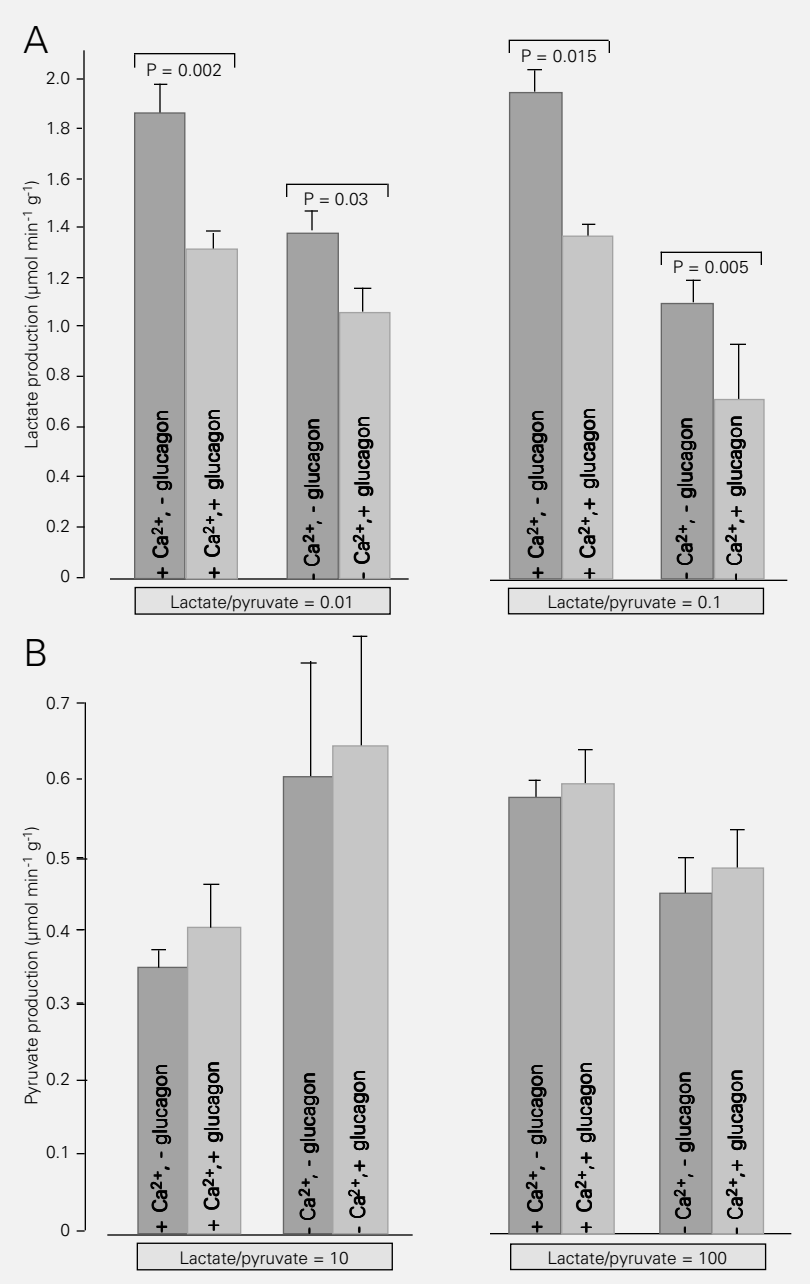


Figure 6 - Influence of glucagon and $Ca^{2+}$ on lactate (A) and pyruvate (B) production. The data were obtained from experiments performed according to the protocol illustrated in Figure 1. Steady-state rates of lactate or pyruvate production in the presence of substrates alone or substrates plus glucagon, corrected for the small basal rates (before substrate infusion), are represented in addition to the corresponding mean standard errors (N = 3 to 5). The substrate concentrations were the following: lactate/pyruvate ratio = 0.01, 0.037 mM lactate and 3.71 mM pyruvate; lactate/pyruvate ratio = 0.1, 0.35 mM lactate and 3.4 mM pyruvate; lactate/pyruvate ratio = 10, 3.5 mM lactate and 0.35 mM pyruvate; lactate/pyruvate ratio = 100, 3.71 mM lactate and 0.037 mM pyruvate. The P values refer to the paired Student *t*-test, which was applied to test the significance of the changes produced by glucagon. Lactate production in $Ca^{2+}$-free perfusion (A) differed from the corresponding controls: P = 0.035 for lactate/pyruvate equal to 0.01 and P = 0.015 for lactate/pyruvate equal to 0.1 (Student *t*-test). No statistically significant differences were observed in the case of pyruvate production (B) (i.e., P>0.05 in all cases).

reveal any clear effect of $Ca^{2+}$ or glucagon. At a lactate/pyruvate ratio equal to 10 there was a tendency toward higher values in the absence of $Ca^{2+}$ as compared to the controls. However, due to the high standard errors, this tendency lacked statistical significance at the 5% level.

## Discussion

The results of the present study revealed that, in the presence of $Ca^{2+}$, stimulation of gluconeogenesis by glucagon increases continuously with the lactate to pyruvate ratio in the range of 0.01 to 100. This variation means a cytosolic $NAD^+$-NADH redox potential range of approximately 280 mV (15). In the absence of $Ca^{2+}$ the maximal action of glucagon was considerably reduced and shifted to a lactate to pyruvate ratio around unity. In the absence of $Ca^{2+}$ and at lactate to pyruvate ratios below unity the action of glucagon on gluconeogenesis became even negative (i.e., inhibitory). It should be emphasized that the basal rates (absence of glucagon) of gluconeogenesis, but not of oxygen uptake and lactate production, were not affected by $Ca^{2+}$ irrespective of the cytosolic $NAD^+$-NADH potential. In the case of gluconeogenesis sensitivity to $Ca^{2+}$ is therefore restricted to the effects of glucagon.

Concerning the response of the gluconeogenic machinery to the cytosolic $NAD^+$-NADH redox potential, Clark and Jarrett (16) showed that the hepatic cyclic phosphodiesterase is inhibited by NADH. Half-maximal inhibition occurs at 20 µM NADH. Other authors have reported that NADH also inhibits adenylate cyclase (26,27). In this case, the concentration for half-maximal inhibition is considerably higher, namely 3 mM (26). These observations may appear contradictory, but, as commented by Rashed and Patel (18), whether cAMP accumulates in any given metabolic condition is ultimately determined by the relative inhibition of the cAMP synthesizing and metabolizing enzymes. Since the intracellular cAMP concentration is higher at higher NADH to $NAD^+$ ratios, it has been proposed that this might be the main reason for the fact that glucagon increases glucose production at high but not at low lactate to pyruvate ratios (16,18). Additionally, it should be noted that Rashed and Patel (18) reported increased glucagon-stimulated $Ca^{2+}$ efflux when livers were perfused with lactate to pyruvate or ß-hydroxybutyrate to acetoacetate ratios similar to those reported for livers of fed rats (usually between 5 and 10). $Ca^{2+}$ movements induced by glucagon thus seem to be favored under reduced conditions.

Groen et al. (28) have shown that a decrease in the cytosolic NADH/$NAD^+$ ratio leads to an increase in phosphoenolpyruvate concentration and thus to a stimulation of the flux through the phosphoenolpyruvate/pyruvate cycle. This cycle transports reducing equivalents from the mitochondria to the cytosol, which are important for gluconeogenesis under oxidized conditions (low lactate to pyruvate ratios). Glucagon inhibits one of the enzymes of this cycle (pyruvate kinase), a property that has been proposed to be coresponsible for the inability of the hormone to increase gluconeogenesis under oxidized conditions (28). It should be added that inhibition of pyruvate kinase is facilitated when $Ca^{2+}$ is present (29). Pyruvate kinase is not the only enzyme directly or indirectly involved in gluconeogenesis that is sensitive to $Ca^{2+}$. At least the following enzymes are known to be stimulated by $Ca^{2+}$-mediated mechanisms: a) Krebs cycle enzymes (13,30), b) pyruvate carboxylase (11), c) phosphoenolpyruvate carboxykinase (31), and d) several protein kinases (12).

Most probably, the experimental patterns obtained in this study result from the interplay of the several factors that have been listed and briefly commented upon above. At low lactate to pyruvate ratios (low NADH to $NAD^+$ ratios), gluconeogenesis is not favored by at least three negative factors: a)

reduced cAMP levels due to the higher phosphodiesterase activity relative to the adenylate cyclase activity (18,26), b) reduced $Ca^{2+}$ movements so that respiration as well as the activity of several key enzymes cannot be increased (18), and c) insufficiency of reducing power in the cytosol due to inhibition of the phosphoenolpyruvate/pyruvate cycle (28). In $Ca^{2+}$-depleted livers perfused with $Ca^{2+}$-free medium and at low lactate to pyuvate ratios the $Ca^{2+}$ movements are probably completely absent. Even the transport of residual $Ca^{2+}$ is unlikely to become stimulated due to the unfavorable conditions in terms of the NADH to $NAD^+$ ratios. The impossibility of $Ca^{2+}$ movements may probably have a strong negative effect on the stimulation of several dehydrogenases (30), making the reducing equivalents less available. This conclusion is corroborated by the fact that lactate production from pyruvate, which represents a loss of reducing equivalents for the cell, was inhibited by glucagon.

As the NADH to $NAD^+$ ratios are gradually elevated by the increasing lactate to pyruvate ratios in the inflowing perfusate, gluconeogenesis is gradually increased by a number of positive events which are more or less the opposite of those occurring at low NADH to $NAD^+$ ratios: a) cAMP accumulates because of the lower phosphodiesterase activity relative to the adenylate cyclase activity (18,26), b) the $Ca^{2+}$ movements are increased so that mitochondrial respiration as well as key enzymes can be adequately stimulated (11,13,18,31), and c) the reducing equivalents are now readily available so that an inhibition of pyruvate kinase even favors gluconeogenesis (28). In $Ca^{2+}$-depleted livers perfused with $Ca^{2+}$-free medium, however, gluconeogenesis is limited by the almost complete absence of $Ca^{2+}$. Although the high NADH to $NAD^+$ ratios favor $Ca^{2+}$ transport, the residual amounts still present in the hepatocytes and in the perfusion fluid are evidently insufficient for full stimulation of respiration and the other $Ca^{2+}$-dependent enzymes involved in gluconeogenesis. Oxygen uptake stimulation seems to be especially limiting under these conditions. As shown in Figure 5, in the absence of $Ca^{2+}$, oxygen uptake stimulation was maximal at a lactate to pyruvate ratio equal to one, with a steady decline for subsequent increases to 10 and 100. With lactate/pyruvate equal to 100 almost no stimulation of oxygen uptake was observed. Gluconeogenesis, however, was almost constant with inflowing lactate/pyruvate between 1 and 100. Apparently, under these specific conditions ($Ca^{2+}$ nearly absent but high NADH to $NAD^+$ ratios) the liver was priorizing gluconeogenesis stimulation by glucagon as much as possible. In this respect, one should recall that in the presence of $Ca^{2+}$ both gluconeogenesis and oxygen uptake stimulation showed the same dependence on the lactate to pyruvate ratio, i.e., the glucose production/extra oxygen consumption ratio was more or less constant. This suggests a good coordination between gluconeogenesis and oxygen uptake in the presence of $Ca^{2+}$, but not in its absence.

Thus, in general terms, it is possible to explain the results obtained in this study in terms of the preexisting literature data. Nevertheless, there are also several details that remain obscure. For example, at low lactate to pyruvate ratios glucagon inhibited gluconeogenesis in the absence of $Ca^{2+}$. This phenomenon, which was associated with inhibition of oxygen uptake, is difficult to explain with the available data. It is also difficult to explain the reasons for the sharp transition between inhibition and stimulation of gluconeogenesis and oxygen uptake when the lactate to pyruvate ratio was increased from 0.1 to 1.0 in the absence of $Ca^{2+}$. In the presence of $Ca^{2+}$ no such sharp transitions were found.

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