CYCLIC AMP INDUCED INHIBITION OF PYRUVATE KINASE FLUX IN THE INTACT LIVER CELL

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Summary: The rate of pyruvate kinase flux in the intact cell is estimated by a new procedure, involving trapping of ^{14}C from $NaH^{14}CO_3$ in a large pyruvate + lactate pool, and calculation of the specific activity of phosphoenol pyruvate. With high concentrations of pyruvate as substrate for isolated rat liver cells, cyclic AMP (0.1 mM) depresses pyruvate kinase flux by about 45%, in addition to inhibiting both glucose and lactate formation. The inhibition of pyruvate kinase may cause an inhibition of hydrogen translocation from the mitochondria to the cytosol.

Introduction

Isotopic evidence for active pyruvate kinase backflow (or "recycling of pyruvate) during gluconeogenesis from pyruvate has been found in rat kidney cortex segments (1) and in perfused rat liver (2). We have proposed (1, 3) that the pyruvate kinase activity is part of a useful cycle when pyruvate is the gluconeogenic substrate, a cycle whose function is an energy-dependent translocation of reducing hydrogen from the mitochondria to the cytosol to provide NADH for the reduction of pyruvate to lactate. The cycle involves the "futile cycle" pyruvate → oxalacetate → phosphoenol pyruvate → pyruvate, but with the addition of the mitochondrial and cytosolic malate dehydrogenases to effect the hydrogen transfer.

Garrison and Haynes (4) and Zahlten et al (5) have shown that glucagon or cyclic AMP causes an unusual inhibition of gluconeogenesis when high concentrations of pyruvate are used as substrate for fasted

rat liver cells, and that the inhibition by cyclic AMP is overcome by the addition of ethanol. Zahlten et al (5) have suggested that the cause of the inhibition may be a glucagon induced depression of pyruvate dehydrogenase activity, in turn causing a reduction of mitochondrial NADH generation.

Methods

Isolated rat liver parenchymal cells from 24 hour fasted animals were prepared essentially by the method of Berry and Friend (6), with the omission of hyaluronidase and substitution of Krebs-Henseleit buffer (7). The cells were incubated in 50 ml Erlenmeyer flasks stoppered with rubber serum caps from which a plastic well was suspended, for 30 minutes at 38° in a rotary shaker set at 150 rev/min. The incubation medium was 5 ml of Krebs-Henseleit buffer containing 100 μmoles of sodium pyruvate, with 5% CO_2/95% O_2 as the gas phase. About 50 μC of $NaH^{14}CO_3$ was used per flask in the 30 minute incubations, or 1 μC of [1-^{14}C] L-alanine in the 5 minute incubations. The $NaH^{14}CO_3$ was injected through the serum cap immediately following addition of the cells and 1 minute gassing with 5% CO_2/95% O_2, while the [1-^{14}C] L-alanine was injected after the cells had incubated for an additional 5 minutes. The incubations were ended by injection of 0.5 ml of 1N H_2SO_4 to the medium and 0.25 ml of 4N NaOH to the hanging center well. $^{14}CO_2$ was collected by 3 hours further shaking. The acidified medium was made to 10 ml and centrifuged. A portion (9 ml) of this was put on a 1 cm x 4 cm column of Amberlite CG-120 (H^+, 100-200 mesh) on top of a 1 cm x 11 cm column of Dowex 1 x 8 (acetate, 100-200 mesh). The columns were washed with water until 35 ml was collected. Glucose was isolated from this neutral fraction by enzymic conversion to glucose-6P (8) and the specific activity determined. The Dowex-1 column was eluted with 0.33 N acetic acid (ketone body fraction); with 2N acetic acid (lactate fraction); and then with 2N formic acid (pyruvate fraction). Glucose, lactate and pyruvate were also determined by enzymic analysis of the acidified medium (9).

Results and Discussion

In order to measure pyruvate kinase flux in the intact cell we have used a new and simpler technique. This involves use of $NaH^{14}CO_3$ (a number of ^{14}C labelled substrates would be suitable) which will label phosphoenol pyruvate prior to labelling of pyruvate. Isolation of the glucose formed will provide a good estimation of the specific activity of phosphoenol pyruvate in fasted liver cells which contain very little glycogen. A very large pool of pyruvate (or lactate) is used, with rather short incubation times and a rather small amount of cells, so that most of the original pool is not used. Thus [1-^{14}C] pyruvate, formed from [1-^{14}C] phosphoenol pyruvate via pyruvate kinase,

will tend to be trapped in the large extracellular pyruvate (+ lactate) pool. For a rigorous estimation, the rates of intracellular pyruvate (or lactate) ⇄ extracellular pyruvate (or lactate) flux and the rate of lactate dehydrogenase would have to be infinite; otherwise a significant proportion of the [1-^{14}C] pyruvate formed in the pyruvate kinase reaction would be further metabolized by pyruvate carboxylase and pyruvate dehydrogenase and not be thoroughly trapped in the large pool. In order to correct for this lack of complete trapping, we use a very short incubation of a substrate (e.g. [1-^{14}C] L-alanine) which will label directly the intracellular pyruvate pool, and determine the partition of label between the pyruvate + lactate pool and other labelled products. Second order correction factors could be developed based on the fact that some (10-15%) of the initial pyruvate was metabolized (to products other than lactate), so that some of the ^{14}C trapped in the pool again left the pool; however these finer adjustments were not attempted.

Table 1 shows that cyclic AMP (0.1 mM) inhibits glucose formation by from 24 to 30% and lactate formation by from 31 to 38% when a high concentration of pyruvate is used as a substrate for fasted rat liver cells. Less than 15% of the pyruvate + lactate pool was utilized in these experiments, in which the initial pool contained 100 μmoles of pyruvate. Dividing the isotopic yield (from $NaH^{14}CO_3$) in the pyruvate and lactate fractions by the phosphoenolpyruvate specific activity (calculated using one-half the specific activity of the glucose formed), gives a measure of pyruvate kinase activity, uncorrected for incomplete trapping. [1-^{14}C] L-Alanine was incubated in parallel flasks, but for a shorter time (5 minutes) to provide a correction for reuse of pyruvate in the cell rather than exchange to the extracellular pool, using the ratio of yields [lactate + pyruvate + glucose + CO_2] / [lactate + pyruvate], neglecting the incorporation of ^{14}C

TABLE 1

Effect of Cyclic AMP on Glucose and Lactate Formation and on Pyruvate Kinase Flux in Rat Liver Cells.

Isolated liver cells from fasted rats (30 mg dry weight in experiment 1 and 22 mg dry weight in experiment 2) were incubated with 20 mM pyruvate plus $NaH^{14}CO_3$ (or [1-^{14}C] L-alanine) as described in the Methods section. The Partition Factor is the ratio of products of metabolism of [1-^{14}C] L-alanine: [lactate + pyruvate + glucose + CO_2] / [lactate + pyruvate]. All rates are μmoles per 30 minute incubation.

Expt.	Cyclic AMP Conc'n (mM)	Glucose Formed (μmoles)	Lactate Formed (μmoles)	PEP sp. act. (cpm/μmole)	^{14}C Yield Lactate Plus Pyruvate (cpm)	Pyruvate Kinase Flux[a] (μmoles)	[1-^{14}C] L-Alanine Partition Factor	Pyruvate Kinase Flux[b] (μmoles)
1	0	4.6	9.8	140,000	893,000	6.4	1.40	8.9
	0.1	3.2	6.1	102,000	369,000	3.6	1.42	5.1
2	0	4.2	7.0	126,000	679,000	5.4	1.36	7.4
	0.1	3.2	4.8	108,000	333,000	3.1	1.29	4.0

[a] Uncorrected.

[b] Corrected by multiplication by [1-^{14}C] L-alanine partition factor.

into amino acids and other organic acids. The partition factors are not greatly affected by cyclic AMP. The isotope results show (a) that cyclic AMP causes a depression of pyruvate kinase flux in the intact cell when high concentrations of pyruvate are used as substrate, and (b) that the rate of pyruvate kinase is quite similar to the rate of lactate formation. The latter result is in accord with our proposal of the hydrogen transfer cycle involving pyruvate kinase. The exact mechanism of the inhibition of gluconeogenesis is not known, but, as shown by Garrison and Haynes (4) and Zahlten et al (5), it presumably involves a depressed cytosolic NADH/NAD^+ ratio, since ethanol reverses the inhibition. Our results suggest that one cause of this lowered NADH/NAD^+ ratio may be inhibition of the mechanism of transfer of reducing hydrogen from the mitochondria to the cytosol.

Taunton et al (10) have shown that glucagon injections *in vivo* cause a rapid decrease in liver pyruvate kinase activity (by assay in the homogenate), while Ljungstrom et al (11) have demonstrated the phosphorylation of rat liver pyruvate kinase by a cyclic AMP stimulated protein kinase. We have carried out preliminary experiments (R. Rognstad, unpublished) on pyruvate kinase fluxes in fasted rat liver cells with lactate as the gluconeogenic substrate. Cyclic AMP stimulates gluconeogenesis from L-lactate, and depresses pyruvate kinase, but the rates of pyruvate kinase are rather low even in the absence of cyclic AMP, so that the major site of action of cyclic AMP under these conditions would appear to be a stimulation of the forward reaction at some site (or sites) rather than a depression of the back reaction at pyruvate kinase.

The new method for estimating pyruvate kinase flux should be suitable for determining whether various agents which affect pyruvate kinase, in cell free systems or as the purified enzyme, also modulate pyruvate kinase flux in the intact cell. Thus alanine and fatty acids are reported to affect pyruvate kinase activity in vitro (12, 13, 14). The effects of changes in diet upon pyruvate kinase flux should also be studied. Here, however, the high rates of glycogen metabolism may make indirect estimation of phosphoenolpyruvate specific activity difficult, and may necessitate isolation of this intermediate.

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