EFFECT OF INSULIN ON ADENYLATE KINASE* ACTIVITY AND GLYCOLYSIS RATE OF THE ISOLATED RAT DIAPHRAGM IN THE ABSENCE OF ADDED GLUCOSE

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1. Introduction

Most energy-converting or energy-coupling processes occur at the expense of ATP which breaks down to AMP and ADP. Since the concentrations of AMP and ATP will be inversely correlated in the intact cell, Atkinson [1] suggested that stimulation by AMP corresponds, in metabolic terms, to negative feed back. Many observations show that both ATP and AMP play important roles in the regulation of energy metabolism not only as substrates and products but also as regulators of enzymatic reactions.

These complex mechanisms of control observed at points of interaction between metabolic pathways, via phosphate acceptors, are illustrated by the effects of adenine nucleotides on phosphofructokinase and the NAD linked isocitrate dehydrogenase: for both enzymes ATP is an inhibitor and this inhibition is relieved by AMP [2–4]. The relative concentrations of ATP and AMP in the tissue will be particularly important: for example, glycolytic rates depend to a large extent on the activity of phosphofructokinase [5] and activation by P_i, AMP and ADP and inhibition by ATP are thought to act as signals for this enzyme to speed up or slow down its activity in response to the demand for high energy phosphate compounds.

This explains why several authors have recently emphasized the importance of activity levels of adenylate kinase in liver and muscle cytosol [6,7] where so many of the adenine-nucleotide mediated processes occur. Adelman et al. [6] have shown that the activity of this enzyme in liver was subject to dietary and hormonal control and particularly sensitive to diabetes and insulin.

The present experiments were designed to explore eventual relationships between early effects of insulin on glycolysis rate [5,8] nucleotide labelling [9] and adenylate kinase activity in subcellular fractions of previously incubated isolated rat diaphragm. An action of the hormone on the enzyme activity in the cytosol is reported. Its relationship with an immediate effect of insulin on glycolysis rates – demonstrated with the aid of monofluorooxaloacetate (F_1OAA) a potent inhibitor of malate dehydrogenase used as a metabolic probe [10] – is considered.

2. Materials and methods

Long Evans male rats were killed by decapitation. The isolated diaphragms were incubated in a Warburg apparatus in Krebs-Ringer bicarbonate medium at 37° in an atmosphere of 95% oxygen plus 5% carbon dioxide in the presence or absence of glucose (2 mg/ml) and insulin (5 μg/ml) over periods from 0 to 120 min. The diaphragms were homogenized in 5 ml of a saline solution containing 150 mM KCl, 50 mM tris, 1 mM dithioerythritol adjusted to pH 7.4. To obtain the cytosol fraction, the homogenate was centrifuged at 100,000 *g* for 60 min and the supernatant recovered. To obtain the mitochondrial fraction the homogenate was centrifuged twice for 10 min at 800 *g* to remove cell debris, then for 10 min at 12,000 *g*. The

* ATP:AMP phosphotransferase EC 2.7.4.3.

pellet was washed three times by resuspension and centrifugation, disrupted in 1% Triton X 100, and centrifuged for 60 min at 100,000 *g*.

The subcellular fractions were incubated for 3 min at 90° in 0.1 N HCl + 1 mM dithioerythritol, then cooled and neutralized. No loss of activity of the adenylate kinase occurred, in agreement with Callaghan [11]. Fractions were chromatographied on Sephadex G-25 equilibrated with the saline solution of the homogenate in order to eliminate nucleotide contamination.

Adenylate kinase activity was assayed spectrophotometrically at 25° as described by Adam [12], in a Gilford multisample absorbance recorder, by measuring ADP formation by following the decrease in absorbance at 340 nm in 3 ml of a reaction mixture containing 33 mM triethanolamine buffer (pH 7.55), 0.15 mM KCl, 10 units of lactate dehydrogenase and pyruvate kinase, 150 μmoles NADH, 3.3 mM phosphoenolpyruvate, 0.2 mM AMP, 3.3 mM ATP (pH 7.55), 3.3 mM $MgCl_2$. The reaction was started by addition of AMP. No activity was found in the absence of AMP, except for small AMP contamination of the commercial ATP. Activities are expressed in units of μmoles of ADP formed per minute. Glutamate dehydrogenase activity in the cytosol, as a measure of mitochondrial dammage during incubation and homogenization, was determined by spectrophotometric analysis of NADH reduction in presence of 10^{-4} M ADP, 10^{-2} M α-ketoglutarate, 0.1 M NH_4^+ and 5×10^{-4} M NADH [13].

For kinetics of the pyruvate accumulation, diaphragms were incubated as described above in the absence or presence of added glucose (2 mg/ml); the reaction was started by tipping F_1OAA or F_1OAA + insulin into the main compartment of the Warburg flask and was stopped at various times by addition of perchloric acid. Enzymatic determinations of pyruvate were performed on aliquots after neutralization according to Bücher et al. [14].

3. Results and discussion

The possible importance of adenylate kinase activity particularly with respect to levels of cytosol AMP has been pointed out by Adelman et al. [6] who found 80% of the enzyme in the liver supernatant, an increased activity in diabetes and decreased activity by insulin treatment or feeding with high concentration of glucose; the mitochondrial enzyme remained unaffected by dietary or hormonal alterations. They found only small amounts of the enzyme in supernatants of heart and skeletal muscle.

Table 1
Variations of adenylate kinase activity in subcellular fractions of rat diaphragm during various incubation time in presence or absence of insulin (units/g diaphragm).

	Cytosol		Mitochondria	
Insulin (5 μg/ml)	0	+	0	+
Incubation time (min)(no. expts.)				
0	132± 8 (3)	142±8 (3)	24±3 (3)	30±4 (3)
30	150±10 (11)	78.4±7 (11)	32±2 (3)	27±3 (3)
120	144± 6 (3)	68±4 (3)	--	--

In contrast to the results of Adelman et al. [6], Lagunas and Sols [7] have recently recovered 80% of the total adenylate kinase in the 100,000 *g* supernatants of intestinal mucosa, heart and skeletal muscle. These results cannot be entirely explained by mitochondrial dammages.

Results of table 1 are in complete agreement with Lagunas and Sols [7]; we recovered about 80% of the rat diaphragm adenylate kinase activity but only 20% of the total glutamate dehydrogenase activity in the cytosol. The mitochondrial adenylate kinase activity is recovered totally only by treatment with Triton X 100 which suggests that it is firmly bound to the mitochondrial membranes. These results do not exclude an inter-membrane enzyme location, as found by Sottocasa et al. [15] and diffusion into the cytosol. When isolated rat diaphragms are incubated with or without added substrate (glucose 2 mg/ml), addition of insulin causes, from the first 30 min of incubation, a rapid decrease of the activity of the supernatant enzyme (48%) and no changes of activity of the mitochondrial enzyme. Adequate controls exclude an action of insulin on the enzymatic coupling system or on the adenylate kinase activity at 0 time. These results suggest an effect of insulin on the supernatant enzyme and not on the solubilization of a mitochondrial-membrane bound adenylate kinase.

In isolated diaphragm, insulin independently stimulates tissue permeability to glucose and metabolic parameters such as glycogen and protein biosynthesis [16]. Stimulation by insulin of incorporation of ^{32}P into energy rich phosphates of the nucleotides, as previously reported [9], may reflect these metabolic changes since we know their role in the activation of sugars, fatty acids [17] and amino acids [18]. The rate of glucose phosphorylation was shown to be an important factor determining the overall rate of glucose uptake when membrane transport was stimulated by insulin in the diaphragm as well as the perfused heart [5,19]. On the basis of changes in the tissue content of glucose-6-phosphate, fructose-6-phosphate and fructose-1-6-diphosphate, as well as pyruvate and lactate formation, it has been proposed that phosphofructokinase is the rate limiting step of glycolysis in muscle [5]. Insulin increases both glycogen biosynthesis via a direct effect on glycogen synthetase [20] and during anaerobic incubation the rate of pyruvate-lactate formation through an enhanced activity of phosphofructokinase [5,8,19]. Fig. 1 illustrates results obtained during short incubations of the isolated rat diaphragm in presence of F_1OAA (10^{-3} M) which selectively blocks ($K_i = 10^{-6}$ M) malate dehydrogenase, a key enzyme of the tricarboxylic acid cycle.

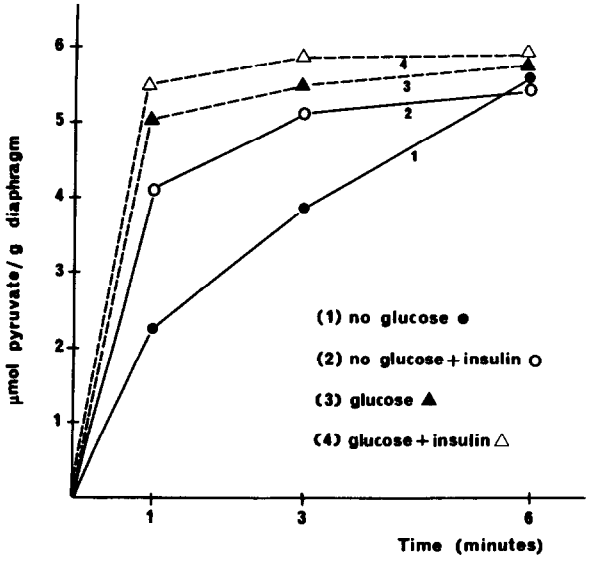

Fig. 1. Rate of pyruvate accumulation in the isolated rat diaphragm incubated with F_1OAA (10^{-3} M) or F_1OAA + insulin (5 µg/ml) in the presence or absence of external glucose (2 mg/ml).

F_1OAA provokes a rapid accumulation of pyruvate (10 to 20 times that of the control, incubated without the inhibitor) and the maximum level reached is independent of the presence of glucose in the incubation medium*. From the first min, insulin provokes a marked increase (+85%) of the pyruvate content of diaphragms incubated in the absence of added glucose and a plateau is reached at the 3rd min. In the absence of insulin, the accumulation is much slower and the maximum level is reached in 6 min. In presence of glucose, the accumulation of pyruvate is so fast that under these conditions the phenomenon may have been overlooked. Insulin therefore stimulates very rapidly the rate of glycolysis from glycogen in diaphragms incubated in the absence of external glucose and this result may explain the glucose-independent increased turn over of the terminal phosphate of ATP observed under the influence of this hormone. As proposed by Atkinson [1], phosphofructokinase, isocitrate dehydrogenase and citrate synthetase all participate in the regulation of carbohydrate utilization associated with the regeneration of ATP by oxidative phosphorylation. Thus regulation of these enzymes by AMP, ADP and ATP should help to maintain the rate of substrate utilization in step with the metabolic demand for ATP. The course of energy metabolism will be controlled by the intracellular concentration of AMP, ADP and ATP. Consequently the rapid action of insulin, described in the rat diaphragm on the cytosol adenylate kinase activity, may be related to this control system.

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