Insulin and cyclic AMP act at different levels on transcription of the L-type pyruvate kinase gene

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Abstract We previously demonstrated that, in hepatocytes in primary culture, the role of insulin on induction of L-type pyruvate kinase (L-PK) gene expression was mainly to induce glucokinase synthesis, needed for glucose phosphorylation to glucose 6-phosphate. However, we show here that when hepatocytes have been isolated from rats starved for 72 h, glucose and constitutive glucokinase expression was not sufficient to fully stimulate the L-PK promoter, low insulin concentrations being still required. In addition, activation remains sensitive to cAMP inhibition, but cannot be reproduced in the absence of insulin by a competitive cAMP antagonist. We propose that both insulin and cAMP act on expression of the L-PK gene at, at least, two levels: positive and negative regulation of glucokinase gene expression, and more downstream levels.

Key words: L-type pyruvate kinase; Glucose; Transcriptional control; Insulin; Cyclic AMP

1. Introduction

Expression of the L-type pyruvate kinase (L-PK) gene, encoding a key enzyme of the glycolytic pathway, is transcriptionally regulated, positively by glucose and insulin and negatively by glucagon and cAMP. Previous studies have demonstrated that this transcriptional regulation by glucose and hormones could be ascribed to a 42 bp glucose response element (GlRE) [1–5]. We have previously shown that insulin can be replaced by constitutive glucokinase synthesis in hepatocytes in primary culture [6], and that glucose induction of the L-PK gene was independent of insulin in hepatoma cells synthesizing insulin-independent hexokinase isoforms instead of glucokinase [7]. These results suggested that activation of glucokinase gene expression, needed to phosphorylate glucose to glucose 6-phosphate, could be the only role of insulin in the transcriptional response of liver genes to glucose. This hypothesis was strengthened and generalized by the recent results of Ferre et al., showing that expression of a glucokinase transgene, controlled by regulatory sequences of the phosphoenolpyruvate carboxykinase gene, normalized glucose metabolism and gene expression in the liver of diabetic mice [8]. However, we show here that, in addition to its role in activation of the glucokinase gene, insulin at very low concentration could also be required for the L-PK gene to switch over from prolonged transcriptional inhibition to transcriptional activation by glucose. This insulin effect is blocked by cAMP but cannot be mimicked by a cAMP antagonist.

2. Materials and methods

2.1. Construction of plasmids

All plasmid constructions used have been previously described. The (L4L3)−119 PK/CAT construct comprises one copy of the L4L3 fragment, ligated to the 119 proximal base pairs of the L-PK promoter, which include the HNF1 and NF1 binding sites. The −150 PK/CAT construct comprises the HNF1, NF1 and HNF4 binding sites, but is devoid of the L4 element [1]. The glucokinase expression vector is made of a 2.3 kbp fragment of glucokinase cDNA [9], containing the entire coding region, inserted between the CMV promoter and the SV40 polyadenylation signal [7]. As a control expression vector, we used an empty PGEM1 vector, including the CMV promoter and the SV40 polyadenylation signal but devoid of glucokinase cDNA insertion. Accumulation of glucokinase mRNA 24 and 48 h after transfection of the glucokinase expression vector was shown by Northern blot analysis [6]. The protein kinase A (PKA) catalytic subunit expression vectors were a generous gift of R.A. Maurer. These plasmids contain the complete coding sequences of the α, β or β- mutant catalytic subunits of Chinese hamster PKA, under the control of the RSV promoter, and have been extensively characterized elsewhere [10].

2.2. Hepatocyte isolation, transfection, and cell culture conditions

Male Sprague-Dawley rats (180–200 g) starved for 72 h were used in all experiments. Hepatocytes were obtained by the collagenase perfusion method as previously described [11]. 3 million freshly isolated cells were plated in 6 cm dishes, in 3 ml of 199 medium supplemented with 1 μM iodothyronine, 1 μM dexamethasone, and 10% (v/v) dialysed fetal calf serum, replaced 6 h later by 199 medium plus hormones and 3% dialysed fetal calf serum. 24 h after isolation, transfection was performed by the lipofection method, using the DOTAP transfection reagent (Boehringer Mannheim), in 199 medium supplemented with 1 μM iodothyronine and 1 μM dexamethasone. Hepatocytes were transfected as previously described [6], with either 5 μg of the KSV2CAT plasmid or the (L4L3)−119 PK/CAT plasmid, and either 20 ng of the glucokinase expression vector or the indicated amount of the expression vector for PKA catalytic subunits. Cells were then cultured in 199 medium containing tri-iodothyronine, dexamethasone, 5 mM or 25 mM glucose, and different concentrations of either insulin, 8-bromo-cAMP (Sigma), or (Rp)-cAMP(s) (Biolog) for approximately 40 h. Medium was replaced every 24 h.

2.3. CAT assays

Cellular protein extraction and CAT assays were performed as previously described [4]. CAT activity was determined with a Molecular Dynamics Phosphorimager. Results were standardized with respect to the amount of cellular proteins used in the CAT assay, then expressed as a percentage of the KSV2CAT activity measured in the same experiment.

2.4. Northern blot analysis

Total RNA isolation and Northern blots experiments were performed as previously described [2]. Northern blots were hybridized with a 2600 bp rat PEPCK cDNA fragment as a probe [12]. A cDNA fragment encoding a ribosomal protein was used as a standardization probe [13]. Specific bands were quantified by scanning the autoradiographs on a Shimadzu densitometer.

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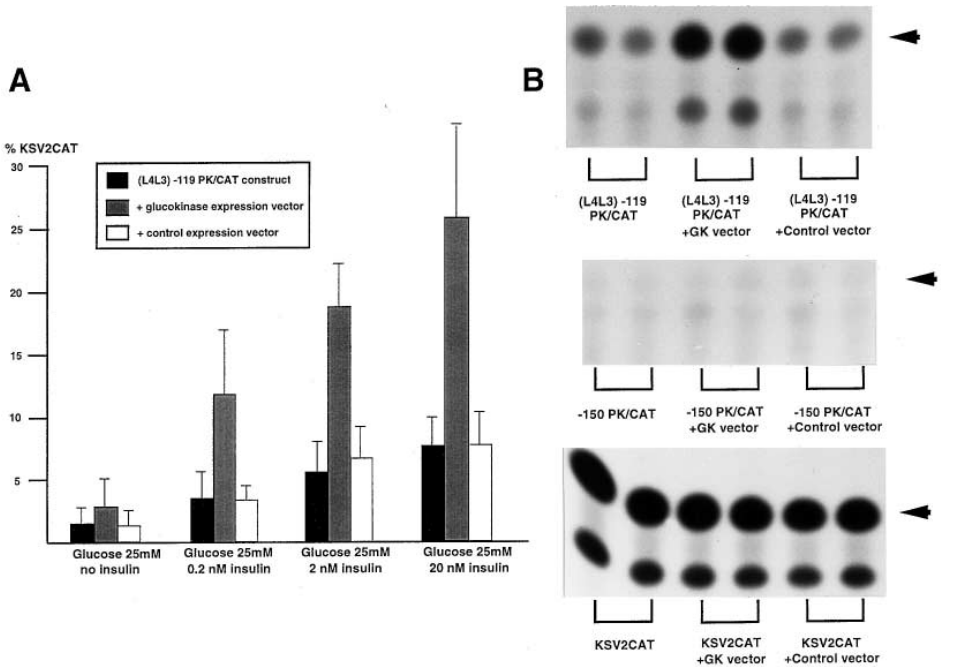

Fig. 1. A: Dose-response curve of insulin effect on the (L4L3)−119 PK/CAT construct cotransfected with the glucokinase expression vector. The (L4L3)−119 PK/CAT reporter was transfected alone (black bars), or cotransfected with the glucokinase expression vector (gray bars), or the control expression vector (open bars). Hepatocytes were cultured in the presence of 25 mM glucose and various insulin concentrations. The CAT activity is expressed as a percentage of the control plasmid KSV2CAT activity. Each value is the mean of three to six experiments; vertical bars indicate S.E.M. B: Effect of the glucokinase expression vector cotransfection on the −150 PK/CAT and KSV2CAT plasmid expression. In this typical experiment, (L4L3)−119 PK/CAT, −150 PK/CAT and KSV2CAT plasmids were either transfected alone, or cotransfected with the glucokinase expression vector or the control expression vector. Hepatocytes were then cultured in the presence of 25 mM glucose and 0.2 nM insulin. Chloramphenicol 3-acetylated forms are indicated by arrows.

3. Results and discussion

3.1. Low insulin concentrations are required for activation of the L-PK promoter by glucose and glucokinase in culture of hepatocytes from starved rats

We examined the effect of constitutive expression of glucokinase on activity of the L-PK promoter in starved rat hepatocytes in primary cultures. Fig. 1A presents the activity generated by the (L4L3)−119 PK/CAT reporter construct in hepatocytes cultured in 25 mM glucose and at various insulin concentrations. When transfected alone, the activity of the reporter plasmid increased as a function of insulin concentration added to the medium, as expected [11]. Cotransfection with a glucokinase expression vector resulted in a poor stimulation of the activity of the (L4L3)−119 PK/CAT construct in the absence of insulin. However, an insulin concentration as low as 0.2 nM led to a substantial activation of the (L4L3)−119 PK/CAT construct in hepatocytes cotransfected with the glucokinase expression vector. As exemplified in Fig. 1A, neither expression of the −150 PK/CAT construct, devoid of the GlRE, nor expression of the KSV2CAT construct was modified by cotransfection with the glucokinase expression vector at the same insulin concentration. These negative results were confirmed by Phosphorimager assays. These controls confirm that activation of the (L4L3)−119PK/CAT plasmid by glucose, insulin and constitutive glucokinase synthesis was dependent on the L-PK GlRE.

The increase of L-PK promoter activity associated with glucokinase overexpression was about 3-fold, practically constant between 2 and 20 nM insulin, conditions under which the endogenous glucokinase gene is transcribed and glucokinase activity is present [14]. This indicates that the amount of active glucokinase is indeed a limiting factor for L-PK gene expression in hepatocytes in primary cultures, since the contribution of exogenous glucokinase is able to further activate the L-PK promoter. Actually, measurement of glucokinase activity in hepatocytes in primary cultures demonstrated that it gradually decreases in the hours following isolation, to a steady-state level of approximately 10% of the initial activity, despite the presence of a very high insulin concentration (1 μM) in the culture medium [15]. Such a limiting role for glucokinase in transcriptional activation of the L-PK gene reinforces the idea that glucokinase plays a permissive role in gene activation by glucose [6]. However, glucokinase induction appears not to be the only role of insulin in activation of these genes, because very low concentrations of insulin remain required for activation of the L-PK promoter in hepatocytes isolated from starved rats and transfected with a glucokinase expression vector. These results are in contrast with those we previously reported in hepatocytes from fed rats, in which glucokinase expression was sufficient to allow for glucose-dependent induction of the L-PK promoter in the total absence of insulin. The difference between the experimental conditions is that the L-PK gene is transcribed in fed rats, but not in starved rats. Therefore, glucokinase plus glucose could be sufficient to maintain active transcription from glucose-responsive promoters, while insulin could be required to shift the system from an inhibited to an activated state. Along the same line, Decaux et al. have previously demonstrated that induction of the L-PK gene by glucose and insulin was very slow when hepatocytes were isolated from starved rats or cultured for a long time without glucose and insulin, and

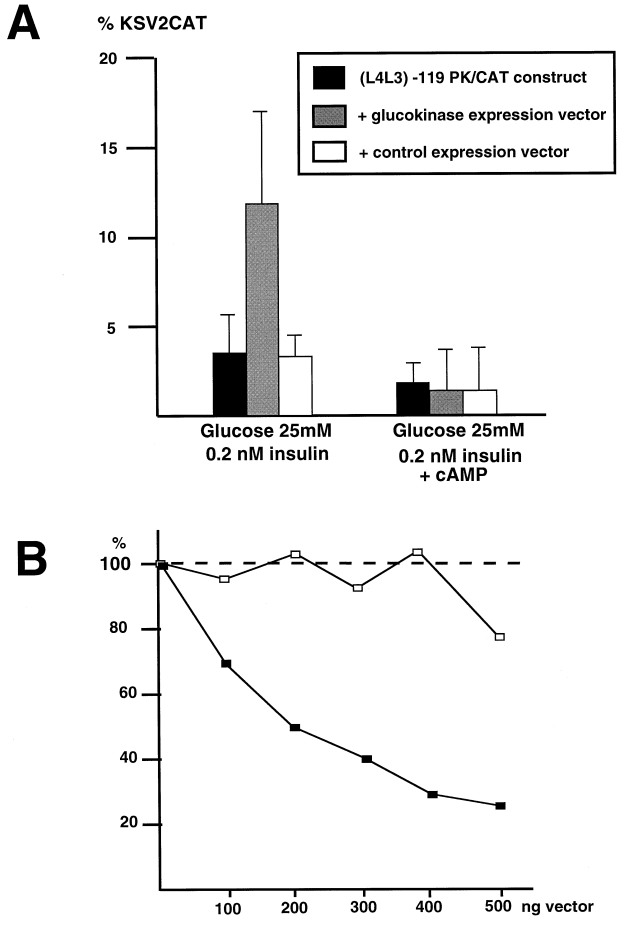

Fig. 2. A: Effect of cAMP analogs on L-PK promoter activity. The (L4L3)−119 PK/CAT construct was either transfected alone (black bars), or cotransfected with the glucokinase expression vector (gray bars) or the control expression vector (open bars). Hepatocytes were cultured in the presence of 25 mM glucose and 0.2 nM insulin, with or without 0.5 mM 8-bromo-cAMP and 0.1 mM 8,4-chlorophenylthio-cAMP. Each value is the mean of four experiments; vertical bars indicate S.E.M. B: Effect of the cotransfection of a catalytic PKA β subunit expression vector on the (L4L3)−119 PK/CAT construct expression. The (L4L3)−119 PK/CAT constuct was cotransfected with increasing amonts of either the PKA β subunit expression vector (dark squares), or the mutant inactive β subunit expression vector (open squares). Hepatocytes were cultured in the presence of 25 mM glucose and 20 nM insulin. Results are expressed as a percentage of the CAT activity found with the reporter construct transfected alone. Each value is the mean of two to five experiments.

rapid when hepatocytes actively transcribing the L-PK gene were depleted of glucose and insulin for 24 h, then restimulated by these agents [11].

3.2. *cAMP inhibits glucose-dependent activation of the L-PK promoter in hepatocytes constitutively synthesizing glucokinase*

We then tested the sensitivity of this additional effect of insulin to cAMP agonists. Fig. 2A shows that cAMP blocks activation of the L-PK promoter in hepatocytes cotransfected with the glucokinase vector and cultured in the presence of glucose and 0.2 nM insulin. Therefore, the action of cAMP in transcriptionally inhibiting the L-PK gene is independent of its recognized negative action on transcription of the endogenous glucokinase gene [14].

It is well known that, in vivo, L-PK gene transcription is inhibited by glucagon within minutes, and that this inhibitory effect can be entirely reproduced by non-hydrolyzable cAMP analogs such as 8-bromo-cAMP [16]. Furthermore, we tested whether this inhibition of the L-PK promoter activity could be reproduced by catalytic subunits of the cAMP-dependent protein kinase (PKA). Fig. 2B presents the activity of the (L4L3)−119 PK/CAT construct in hepatocytes cultured under glucose-insulin conditions and cotransfected with different amounts of expression vectors for either the Chinese hamster PKA β catalytic subunit, or the same β catalytic subunit inactivated by a mutation within its ATP binding site [10]. Expression of the PKA β catalytic subunit in hepatocytes results in a strong inhibition of the reporter construct activity, up to 500 ng. Higher amounts led to non-specific inhibition by 'squelching'. Therefore, not surprisingly, the negative transcriptional action of cAMP on glucose-dependent activation of the L-K promoter is most likely mediated by PKA.

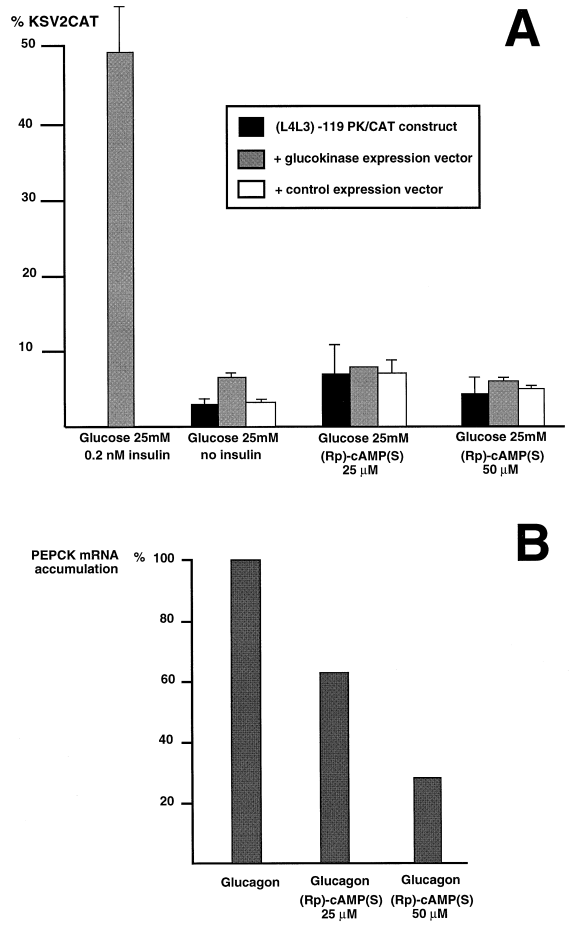

Fig. 3. A: Effect of cAMP competitive antagonists on (L4L3)−119 PK/CAT construct expression. The reporter construct was tranfected either alone, or with the glucokinase expression vector or the control expression vector. Transfected hepatocytes were cultured with 0.2 nM insulin as a positive control, or with two different concentrations of (Rp)-cAMP(s) (Biolog). Each value is the mean of three experiments. B: Effect of cAMP competitive antagonists on the PEPCK mRNA accumulation induced by glucagon in hepatocytes in primary culture. Hepatocytes were either cultured in 1 μM glucagon, or preincubated for 30 min with different concentrations of (Rp)-cAMP(s), then cultured in 1 μM glucagon. 30 μg of total RNA was loaded in each well. Relative signal intensity is indicated here after standardization in relation to the R45 control probe.

3.3. *Inhibition of the cAMP-activated pathway is not sufficient to replace insulin in the activation of the L-PK promoter*

As insulin is known to counteract effects of the cAMP pathway, by acting at several levels [17–19], we addressed the question whether a blockade of the endogenous cAMP pathway could suppress the insulin requirement in our model, or even replace glucose in stimulating L-PK promoter. For this purpose, we used an adenosine cyclic 3′,5′-phosphorothioate, (Rp)-cAMP(s), which acts as a competitive antagonist of cAMP on the mammalian PKA [20]. Fig. 3A shows that (Rp)-cAMP(s) at 25 or 50 μM could not replace a low concentration of insulin in allowing L-PK promoter activation by glucose and glucokinase. Higher concentrations of (Rp)-cAMP(s) induced significant cell death, and therefore could not be used. Nevertheless, efficacy of (Rp)-cAMP(s) at a 50 μM concentration was verified by Northern blot experiments, showing a strong inhibition of the phosphoenolpyruvate carboxykinase mRNA accumulation in hepatocytes cultured in the presence of glucagon (Fig. 3B). Such an inhibitory effect on cAMP-dependent transcriptional activation confirms results previously described for the tyrosine aminotransferase gene in hepatocytes in primary culture [21].

This result indicates that, despite the presence of active glucokinase, a blockade of the cAMP pathway is not sufficient by itself to allow activation of the L-PK gene promoter, and suggests therefore that insulin should exert at least one more positive action to allow the glucose signal to be transmitted to the transcriptional machinery.

As a first hypothesis, insulin might remain necessary to induce expression of, or to activate, other enzymes involved in glucose metabolism. Insulin is known to be involved in the short-term and long-term regulation of several other enzymes of the glycolytic pathway [22], and, in the pentose phosphate pathway, is involved in the transcriptional activation of the key enzyme glucose 6-phosphate dehydrogenase [23,24]. A puzzling element, however, is the low concentration of insulin (0.2 nM) sufficient to achieve expression of the L-PK gene when active glucokinase is already present. This extremely low concentration is not consistent with an action of insulin upon enzymes of the glucose metabolism, since insulin concentrations required to activate these enzymes are usually much higher, especially in hepatocytes in primary cultures.

Alternatively, insulin might initiate a phosphorylation-dephosphorylation cascade, leading to a modification of the phosphorylation status permissive for the glucose-dependent activation of the L-PK promoter. One can speculate that upon prolonged arrest of transcription of the L-PK gene, PKA-dependent or -independent processes might affect either directly or indirectly the transcription factors and other proteins constituting the glucose response complex. As a logical consequence, a reverse dephosphorylation process should be necessary to allow transcriptional activation. The steps where insulin could interfere along the glucose signal pathway are now under investigation in our laboratory. For this purpose, starved rat hepatocytes in primary culture transfected with a glucokinase constitutive expression vector provide an attractive, physiologically relevant model, which will allow us to test the capacity of candidate phosphatases or enzymes, synthesized under direction of cotransfected expression vectors, to make insulin totally dispensable for the activation of the L-PK promoter.

In conclusion, we establish in this paper that both insulin and cAMP act on the transcription of the L-PK gene at different levels. The first is positive and negative regulation of the endogenous glucokinase gene transcription, required to phosphorylate glucose to glucose 6-phosphate. In addition, one or more downstream steps require low concentrations of insulin to allow glucose activation of the L-PK promoter when transcription has been blocked for a long time.

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