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Regulation of mitochondrial pyruvate carboxylation in isolated hepatocytes by acute insulin treatment

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The effect of acute insulin treatment of hepatocytes on pyruvate carboxylation in both isolated mitochondria and cells rendered permeable by filipin was examined. Challenging the cells with insulin alone had no effect on either the basal rate of pyruvate carboxylation or gluconeogenesis, although it did suppress the responses to both glucagon and catecholamines. Insulin treatment was unable to antagonize the enhanced rate of pyruvate carboxylation caused by stimulation of the cells with either angiotensin or vasopressin. Neither insulin nor the gluconeogenic hormones altered the total extractable pyruvate carboxylase activity in the isolated mitochondria, suggesting that the effect of hormones at the level of the isolated intact organelle was mediated via alterations in the intramitochondrial concentrations of effector molecules, notably ATP and the [ATP]/[ADP] ratio and substrate availability. The alterations in pyruvate carboxylation correlate well with glucose synthesis in terms of sensitivity to effector molecules, putative second messengers and time of onset of the response, indicating that alterations in the flux through this enzyme are compatible with it being an important site in the control of gluconeogenesis from C_3 precursors.

It is well established that insulin plays a major role in the regulation of gluconeogenesis, antagonizing the action of the gluconeogenic hormones glucagon and adrenaline (Exton *et al.*, 1971; Pilkis *et al.*, 1975; Claus & Pilkis, 1976; Hue *et al.*, 1978). Analyses of the concentrations of intermediary metabolites in rat livers perfused with pyruvate and lactate have led to the conclusion that a prime locus at which glucagon, adrenaline and insulin act to control glucose synthesis occurs within the sequence of reactions responsible for the conversion of pyruvate into phosphoenolpyruvate (Exton & Park, 1969; Williamson *et al.*, 1969; Mallette *et al.*, 1969). Although it is established that the regulation of pyruvate kinase may be important in this respect (for review, see Claus & Pilkis, 1981), there is now evidence to suggest that a dual mechanism may operate and that, in addition, acute control of pyruvate carboxylase may be pertinent. This conclusion has arisen indirectly from measurements of futile cycling (Rognstad & Katz, 1977) and also from measurements of metabolic intermediates in hepatocytes treated with glucagon, which have indicated that the site of stimulation resides at the level of the mitochondria (Siess *et al.*, 1977). Direct measurements of the rate of pyruvate carboxylation both in the isolated mitochondria (Adam & Haynes, 1969; Garrison & Haynes, 1975; Garrison & Borland, 1979; Titheradge *et al.*, 1979) and in hepatocytes rendered permeable with the polyene antibiotic filipin (Allan *et al.*, 1983) have indicated there is an excellent correlation between the alterations in enzyme activity upon treatment of cells with gluconeogenic hormones and that of gluconeogenesis, in terms of time of onset of the response, sensitivity and selectivity towards agonists.

Although considerable efforts have been expended to establish a role for pyruvate carboxylase in the stimulation of gluconeogenesis, no data are available as to the acute effects of insulin at this level, and before it can be considered seriously the enzyme must be shown to be sensitive to insulin treatment. Therefore the object of this study was to examine the effect of acute insulin treatment of isolated hepatocytes on pyruvate carboxylase activity, and to correlate alterations in CO_2 fixation both by the isolated mitochondria and by cells made permeable with filipin with that of glucose synthesis.

Experimental

Materials

Type IV collagenase, bacitracin, [arginine]vasopressin, angiotensin, phenylephrine hydrochloride,

adrenaline bitartrate, bovine serum albumin (fraction V) and sodium pyruvate were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). [2-^{14}C]Pyruvate and [4-^{14}C]aspartate were obtained from Amersham International (Amersham, Bucks., U.K.). Glucagon and insulin were gifts from Novo Laboratories (Basingstoke, Hants., U.K.). Filipin was given by Upjohn Ltd. (Crawley, Sussex, U.K.). All other chemicals were AnalaR grade from BDH Chemicals (Enfield, Middx., U.K.).

Methods

Fed male Sprague–Dawley rats weighing 180–240 g were anaesthetized by intraperitoneal injection of sodium pentobarbital (60 mg/kg). Hepatocytes were prepared by a modification of the procedure of Berry & Friend (1969) essentially as described by Hutson *et al.* (1976), except that washed aged erythrocytes were omitted from the perfusion medium. The hepatocytes were resuspended in Krebs–Ringer bicarbonate buffer, pH 7.4 (Krebs & Henseleit, 1932), containing 1.5% gelatin, 2 mM-glucose and 0.1% (w/v) bacitracin. The final protein concentration was approx. 15 mg/ml.

For the preparation of mitochondria, 10 ml of the cell suspension was preincubated for 25 min before the addition of insulin (50 nM) or vehicle. After a further 5 min of incubation, glucagon, catecholamines, vasopressin or angiotensin were added to the final concentration indicated, together with a lactate/pyruvate mixture to give final concentrations of 2.5 mM-pyruvate and 2.5 mM-lactate. The incubation was continued for a further 10 min after addition of the gluconeogenic hormones (or as indicated) and mitochondria prepared as described previously (Allan *et al.*, 1983).

The rate of gluconeogenesis was determined in an incubation volume of 1.6 ml with [2-^{14}C]pyruvate (sp. radioactivity 50 μCi/mmol) in the pyruvate/lactate mixture. [^{14}C]Glucose output was measured as described by Exton & Park (1967), except that Amberlite IRA-68 replaced Duolite A-4 as the anion-exchange resin.

Mitochondrial pyruvate carboxylation was assayed by the incorporation of $NaH^{14}CO_3$ into acid-stable products, as described by Adam & Haynes (1969). Determination of the rate of pyruvate carboxylation in isolated hepatocytes was performed as described previously by using cells treated with 100 μM-filipin (Allan *et al.*, 1983), and total pyruvate carboxylase activity was measured in mitochondrial extracts after freeze–thawing by the method of Weinberg & Utter (1979). ATP and ADP were measured by bioluminescence with an ATP-monitoring reagent from LKB Wallac (Turku, Finland) after conversion of the ADP into ATP (Lowry *et al.*, 1964). Cyclic AMP was determined in the cells plus medium as described by Brooker *et al.* (1979), and acetyl-CoA by its conversion into [^{14}C]citrate (Prinz *et al.*, 1966). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V) as the standard.

Results are expressed as means ± S.E.M., with the numbers of different cell preparations given in parentheses.

Results and discussion

Effect of insulin treatment of isolated hepatocytes on the rate of pyruvate carboxylation in subsequently isolated mitochondria

Previous studies have shown that mitochondria isolated from hepatocytes treated with glucagon, adrenaline, vasopressin or angiotensin display enhanced rates of pyruvate carboxylation when compared with those isolated from control cells (Garrison & Haynes, 1975; Garrison & Borland, 1979; Titheradge *et al.*, 1979; Allan *et al.*, 1983), and from this it has been suggested that pyruvate carboxylase might be an important locus in the conversion of pyruvate into phosphoenolpyruvate and hence gluconeogenesis. The present study has examined the effect of treatment of hepatocytes with insulin on the regulation of pyruvate carboxylase activity and gluconeogenesis, to determine whether this would be compatible with the above concept.

Table 1 shows the effect of 50 nM-insulin on the rate of pyruvate carboxylation in mitochondria isolated from control and hormone-treated cells. Addition of maximal concentrations of glucagon, adrenaline, phenylephrine or the vasoactive peptides angiotensin or vasopressin enhanced the rate of pyruvate carboxylation over a 10 min incubation period of the cells with the hormone, in agreement with previous studies (Garrison & Haynes, 1975; Garrison & Borland, 1979; Titheradge *et al.*, 1979; Allan *et al.*, 1983). Pretreatment of the cells with insulin 5 min before the addition of the gluconeogenic hormones resulted in a significant suppression of the response of the cells to glucagon, adrenaline and the α-adrenergic agonist phenylephrine, but no effect was apparent on the enhanced rate caused by the vasoactive peptides, nor was there any significant effect when the cells were incubated with insulin alone. These results are in good agreement with the documented literature of the effects of insulin on gluconeogenesis after treatment of hepatocytes with gluconeogenic hormones, as numerous studies have indicated that insulin can antagonize the action of both glucagon and catecholamines (Exton *et al.*, 1971; Claus & Pilkis, 1976; Feliu *et al.*, 1976), and Whitton *et al.* (1978) have shown that insulin is unable to suppress the increase in gluconeogenesis caused by the presence of vasopressin or angiotensin.

Table 1. *Effects of insulin on the rate of pyruvate carboxylation in intact mitochondria and the total pyruvate carboxylase activity*

Hepatocytes were incubated and mitochondria prepared as described in the Experimental section. Insulin was added to the cells to a final concentration of 50nM 5min before the addition of the other hormones. Rates of pyruvate carboxylation in intact mitochondria and pyruvate carboxylase activity in mitochondrial extracts were assayed by the methods of Adam & Haynes (1969) and Weinberg & Utter (1979) respectively. Results are expressed in nmol/min per mg of mitochondrial protein and are the means ± S.E.M. for six different cell preparations. $^{**}P < 0.001$ compared with control; $^{\dagger}P < 0.01$ compared with the rate in the absence of insulin.

	Pyruvate carboxylation		Pyruvate carboxylase activity	
Additions	No insulin	+ Insulin	No insulin	+ Insulin
Vehicle	15.3 ± 1.7	16.2 ± 1.6	103.6 ± 5.7	98.6 ± 5.2
Glucagon (1 nM)	28.9 ± 1.7**	20.5 ± 1.8†	106.3 ± 6.7	107.9 ± 5.9
Adrenaline (100 nM)	30.7 ± 1.6**	23.1 ± 1.6†	108.5 ± 8.9	106.1 ± 7.1
Phenylephrine (100 nM)	25.8 ± 1.3**	18.9 ± 1.2†	110.2 ± 7.7	110.1 ± 5.6
Angiotensin (1 μM)	32.8 ± 2.5**	30.3 ± 2.1	101.9 ± 8.0	99.2 ± 7.7
Vasopressin (1 munit/ml)	28.6 ± 1.6**	27.7 ± 1.6	106.7 ± 8.0	99.6 ± 7.8

Table 2. *Effects of insulin on the rate of pyruvate carboxylation in filipin-treated cells and cyclic AMP production*

The experimental protocol was as described in Table 1. At 2min after the addition of the gluconeogenic hormones, samples were removed for the determination of cyclic AMP and the incubation was continued for a further 8min before the rate of pyruvate carboxylation by the cells was determined in the presence of 100μM-filipin. The final insulin concentration was 50nM. Results are the means ± S.E.M. for six different cell preparations. $^{**}P < 0.001$ for difference from controls; $^{\dagger}P < 0.01$ for difference from rate in the absence of insulin.

	Pyruvate carboxylation (nmol/min per mg of cell protein)		Cyclic AMP production (pmol/mg of cell protein)	
Additions	No insulin	+ Insulin	No insulin	+ Insulin
Vehicle	11.3 ± 1.0	11.3 ± 1.0	2.31 ± 0.19	2.04 ± 0.18
Glucagon (1 nM)	17.8 ± 1.3**	13.7 ± 1.3†	24.83 ± 2.48**	11.04 ± 2.27†
Adrenaline (100 nM)	17.5 ± 1.0**	14.1 ± 1.6†	4.04 ± 0.96	2.88 ± 0.68
Phenylephrine (100 nM)	16.4 ± 0.8**	13.2 ± 1.2†	2.54 ± 0.39	2.39 ± 0.42
Angiotensin (1 μM)	17.4 ± 1.7**	16.7 ± 2.0	2.13 ± 0.18	2.02 ± 0.16
Vasopressin (1 munit/ml)	16.4 ± 1.7**	14.8 ± 1.8	2.14 ± 0.25	2.03 ± 0.23

Table 1 also shows that acute treatment with the above hormones has no significant effect ($P > 0.1$) on the total pyruvate carboxylase activity within the mitochondria as measured in extracts treated with Triton X-100. The enzyme activity expressed in intact mitochondria prepared from control and hormone-treated cells was approx. 15% and 30% respectively of the maximal activity, indicating that the enzyme was severely restrained within the mitochondrial matrix. The effect of both the gluconeogenic hormones and insulin was to alter the degree of restraint, possibly as a result of alterations in substrate availability and the concentration of mitochondrial effector molecules. This agrees with the original observations by Adam & Haynes (1969) for both glucagon and adrenaline, although the total pyruvate carboxylase activity measured in the present study is substantially higher than in their work. This is probably the result of the improved assay technique described by Weinberg & Utter (1979).

Effect of insulin on cyclic AMP concentrations and the rate of pyruvate carboxylation in filipin-treated hepatocytes

We previously demonstrated that the ability of gluconeogenic hormones to stimulate pyruvate carboxylase activity is not restricted to the isolated mitochondria, but is also apparent when rates of pyruvate carboxylation are measured in mitochondria *in situ*, in cells made permeable by treatment with the polyene antibiotic filipin (Allan *et al.*, 1983). Table 2 shows the effect of insulin pretreatment of the cells for 15min, in the presence and absence of gluconeogenic hormones, on the rate of pyruvate carboxylation measured in hepatocytes in the presence of 100μM-filipin. The effect of insulin on CO_2 fixation measured *in situ* was qualitatively the same as that determined in the isolated mitochondria, in that insulin pretreatment alone did not significantly alter the incorporation of $^{14}CO_2$ into acid-stable products, although it did decrease the effect of glucagon, adrenaline and phenylephrine. It

was also unable to inhibit the response caused by angiotensin or vasopressin. These results confirm the hypothesis that alterations in the rate of CO_2 fixation measured in the isolated mitochondria reflect physiological changes in activity of pyruvate carboxylase within the cell and are not merely artifacts introduced by the mitochondrial isolation procedure. The degree of stimulation by the gluconeogenic hormones in the filipin-treated cells was lower than that measured in the isolated mitochondria, suggesting that some enhancement of the effect of the hormones may have occurred during isolation, as described by Siess *et al.* (1981). However, the effects were still of sufficient magnitude to support the concept that pyruvate carboxylase exhibits all the characteristics required for it to be considered a major point of regulation of glucose synthesis.

Measurements of cyclic AMP production by the cells 2min after introduction of the gluconeogenic hormones confirm data from previous studies, since the increase in cyclic AMP coincides with the stimulation of pyruvate carboxylation after glucagon treatment, but not that with adrenaline or phenylephrine (Garrison & Haynes, 1975; Garrison & Borland, 1979; Titheradge *et al.*, 1979). A small increase in cyclic AMP was observed with 100mM-adrenaline, but this was insignificant over the series. Similarly, angiotensin and vasopressin did not alter cyclic AMP synthesis despite increasing the rate of pyruvate carboxylation. Cyclic AMP formation by the cells was unaltered by treatment with insulin either alone or in combination with phenylephrine, angiotensin or vasopressin; however, it produced a marked decrease in the elevation of cyclic AMP concentration caused by glucagon and completely abolished the small increase caused by adrenaline. These alterations in cyclic AMP concentration and pyruvate carboxylase activity correlate well with the known effects of insulin action on gluconeogenesis in terms of the second messenger involved in the response (Exton *et al.*, 1971; Pilkis *et al.*, 1975; Claus & Pilkis, 1976; Hue *et al.*, 1978; Hems *et al.*, 1978), and suggest that three distinct mechanisms may exist for the stimulation of both pyruvate carboxylation and gluconeogenesis: first, the cyclic AMP-dependent mechanism characteristic of glucagon; second, the cyclic AMP-independent α-adrenergic system of the catecholamines; and finally, the vasopressin and angiotensin mechanism, which is also cyclic AMP-independent but resistant to the effects of insulin. It is apparent that the effects of both the catecholamines and vasoactive peptides are mediated by alterations in Ca^{2+} flux (Van de Werve *et al.*, 1977; Assimacopoulos-Jeannet *et al.*, 1977; Hems *et al.*, 1978; Whitton *et al.*, 1978; Hue *et al.*, 1978; Garrison *et al.*, 1979), and a mobilization of mitochondrial Ca^{2+} has been implicated in this process (Blackmore *et al.*, 1979; Babcock *et al.*, 1979; Taylor *et al.*, 1980). However, it is still unclear whether a different mechanism and second messenger is involved in the action of the two types of agonist, or whether the effect of insulin is at the level of the receptor rather than the second messenger (Dehaye *et al.*, 1981).

Effect of insulin on the time of onset of the stimulation of mitochondrial pyruvate carboxylation and gluconeogenesis in hepatocytes treated with glucagon and adrenaline

Fig. 1 shows the effect of preincubation of the cells with 50nM-insulin on the time course of the stimulation of gluconeogenesis and mitochondrial pyruvate carboxylation by 0.2nM-glucagon. After the addition of glucagon there was a parallel stimulation of the rate of CO_2 fixation with that of gluconeogenesis, which was preceded in the cells by a rise in the concentration of cyclic AMP. Insulin alone had no effect on any of these parameters, nor was it able to prevent completely the increase in either pyruvate carboxylation or gluconeogenesis caused by glucagon as a result of the breakthrough of cyclic AMP production in the insulin-treated cells within the first 2min. After 2min all the time points showed a suppression by insulin, although the effect was most marked at later times, where the response to glucagon was terminated more rapidly in the insulin-treated cells.

Similar results were obtained after treatment of the cells with 30nM-adrenaline (results not shown), although the time of onset of the increase in CO_2 fixation and gluconeogenesis was more rapid, being highly significant within 2min. In addition, the effect of insulin was also apparent within 2min, in contrast with the effect in the presence of glucagon. The rapid response to catecholamines is in agreement with previous studies, and with the concept that glucagon and catecholamines act to stimulate gluconeogenesis and pyruvate carboxylation by two independent mechanisms (Garrison & Borland, 1979; Titheradge *et al.*, 1979).

Sensitivity of hepatocytes to glucagon and adrenaline in the presence of insulin

Previous studies have indicated that there is an excellent correlation between the sensitivity of the rate of mitochondrial pyruvate carboxylation and gluconeogenesis after treatment of hepatocytes with glucagon and adrenaline (Garrison & Haynes, 1975; Garrison & Borland, 1979; Titheradge *et al.*, 1979). Figs. 2 and 3 show the effect of a 5min preincubation period with insulin on the stimulation of glucose synthesis and mitochondrial CO_2 fixation with increasing concentrations of both glucagon and adrenaline; 50nM-insulin was an effective antagonist to the response of both glucagon and adrenaline over the concentration ranges 10pM–1nM and 10–100nM respectively. The slight differences in the sensitivity

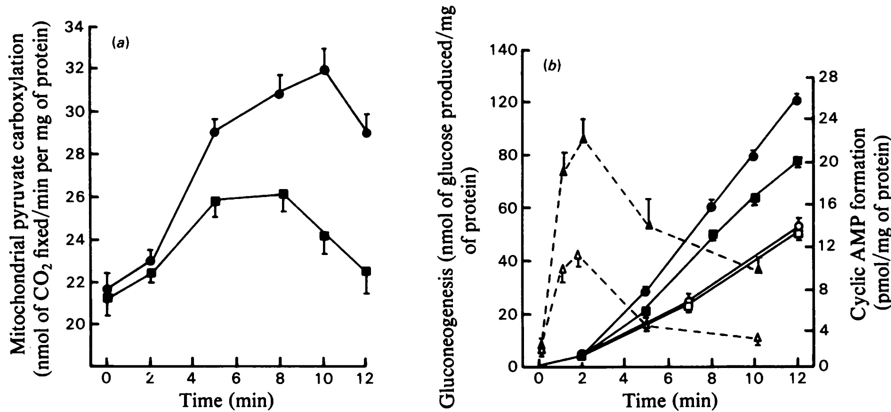

Fig. 1. *Effect of insulin on the time of onset of the stimulation of mitochondrial pyruvate carboxylation (a) and cellular glucose and cyclic AMP formation (b) after treatment with glucagon*

Cells were preincubated as described in the Experimental section. Insulin (50nM) was added 5min before the addition of glucagon (0.2nM). Samples were removed for the determination of the rate of glucose synthesis and cyclic AMP formation and for preparation of mitochondria at the times indicated. Each time point is the mean ± S.E.M. for five different cell preparations. ●, Pyruvate carboxylation and glucose synthesis in the presence of glucagon; ■, pyruvate carboxylation and glucose synthesis in the presence of glucagon plus insulin; ○, glucose synthesis with vehicle alone; □, glucose synthesis in the presence of insulin alone; ▲, cyclic AMP in the presence of glucagon; △, cyclic AMP in the presence of glucagon plus insulin.

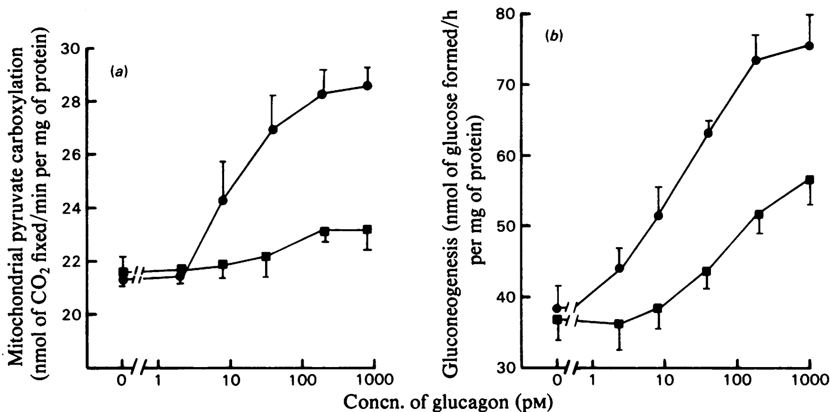

Fig. 2. *Effect of insulin on the dose–response of mitochondrial pyruvate carboxylation (a) and glucose synthesis (b) to glucagon*

Cells were incubated as described in the Experimental section. Insulin (50nM) was added 5min before the addition of glucagon at the concentrations indicated. After the addition of glucagon, the incubation was continued for a further 10min, and the rate of gluconeogenesis was determined over this period, before the mitochondria were prepared and pyruvate carboxylation was assayed. The results shown are means ± S.E.M. for five different cell preparations. ●, Glucagon alone; ■, glucagon plus insulin.

of pyruvate carboxylation and gluconeogenesis to insulin could be due to the experimental design, since the measurement of glucose synthesis was the result of an accumulation of [^{14}C]glucose over the 10min incubation period, whereas the rate of pyruvate carboxylation was a measure of the activity of the enzyme as it existed at the end of the 10min period.

Effect of insulin on mitochondrial acetyl-CoA, ATP and ADP concentrations

The data presented in Table 1 indicate that pyruvate carboxylase activity is under considerable metabolic restraint within the mitochondrial matrix and that the action of the gluconeogenic hormones could be to relieve this restraint and increase the flux

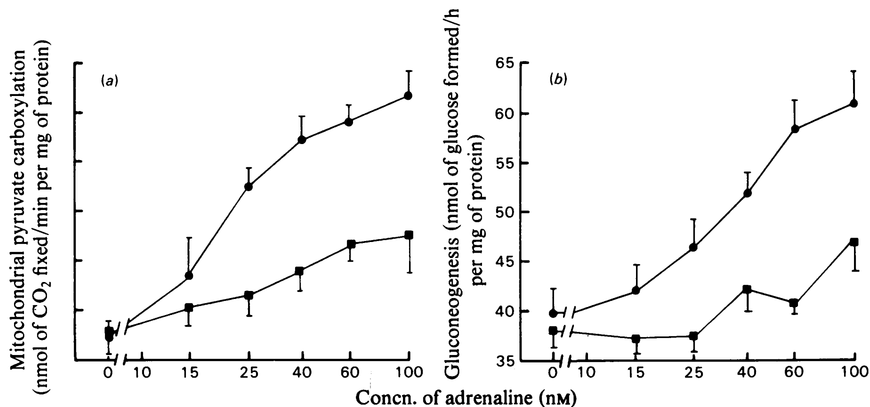

Fig. 3. *Effect of insulin on the dose–response of mitochondrial pyruvate carboxylation* (*a*) *and glucose synthesis* (*b*) *to adrenaline*

Details are as for Fig. 2, except that adrenaline replaced glucagon at the concentrations indicated. ●, Adrenaline alone; ■, adrenaline plus insulin.

Table 3. *Effect of insulin on the mitochondrial concentrations of acetyl-CoA, ATP and ADP*

Hepatocytes were incubated and mitochondria prepared as described in the Experimental section. The mitochondria were incubated under the conditions used for the assay of pyruvate carboxylation and the incubation was terminated after 5 min by the addition of $HClO_4$ (final concn. 0.26 M). After neutralization with $KHCO_3$, acetyl-CoA and adenine nucleotides were assayed as described in the Experimental section. Insulin was added to the cells to a final concentration of 50 nM. Results are expressed as nmol/mg of mitochondrial protein and are the means ± S.E.M. for six different cell preparations. $^*P < 0.05$ and $^{**}P < 0.001$ compared with control; $^†P < 0.05$ compared with value in absence of insulin.

Additions	Insulin	Acetyl-CoA	ATP	ADP	[ATP]/[ADP]
Vehicle	−	2.83 ± 0.28	8.11 ± 0.76	2.38 ± 0.49	3.43 ± 0.42
	+	2.73 ± 0.14	8.58 ± 1.24	2.24 ± 0.31	3.96 ± 0.46
Glucagon (1 nM)	−	3.14 ± 0.24	10.66 ± 0.88*	2.08 ± 0.32	5.02 ± 0.41*
	+	3.15 ± 0.28	9.14 ± 1.22†	2.59 ± 0.60	3.41 ± 0.42†
Adrenaline (100 nM)	−	3.64 ± 0.16*	12.89 ± 0.89**	2.74 ± 0.52	4.71 ± 0.53*
	+	3.16 ± 0.25	10.49 ± 1.12†	2.79 ± 0.31	3.63 ± 0.34†

of pyruvate through the enzyme. The enzyme is known to be sensitive to a number of metabolic intermediates, including acetyl-CoA (Keech & Utter, 1963), ATP and the [ATP]/[ADP] ratio (Stucki *et al.*, 1972; von Glutz & Walter, 1976), glutamate (Scrutton & White, 1974), pyruvate (McClure & Lardy, 1971) and Ca^{2+} ions (McClure & Lardy, 1971; Wimhurst & Manchester, 1970). Experiments to characterize which of these may be of importance in the mechanism of action of glucagon and catecholamines have suggested that the net result is a summation of alterations in many of them, as hormone treatment has been demonstrated to increase rates of pyruvate uptake into the mitochondria (Adam & Haynes, 1969; Titheradge & Coore, 1976*a*,*b*; Halestrap, 1978; Martin & Titheradge, 1983), the intramitochondrial content of ATP and the [ATP]/[ADP] ratio (Siess *et al.*, 1977; Bryla *et al.*, 1977; Prpić *et al.*, 1978; Titheradge *et al.*, 1979; Titheradge & Haynes, 1980) and the concentration of acetyl-CoA (Adam & Haynes, 1969; Siess *et al.*, 1977), and to decrease the intramitochondrial concentration of glutamate (Siess *et al.*, 1977).

Table 3 shows the effect of treatment of hepatocytes with insulin on acetyl-CoA, ATP and ADP concentrations and the [ATP]/[ADP] ratio in deproteinized extracts of mitochondria incubated under the conditions used for the assay of pyruvate carboxylation. Mitochondria prepared from cells treated with maximal concentrations of glucagon and adrenaline showed a slight elevation in their acetyl-CoA content above that of the control, although this was only significant with adrenaline. The effect of adrenaline was reversed by prior incubation of the cells with insulin, although insulin had no effect on either the basal acetyl-CoA concentrations or the small stimulation apparent in

the presence of glucagon. These data indicate that, although acetyl-CoA may be important in the control of pyruvate carboxylase activity in the isolated mitochondria after adrenaline treatment, it is unlikely to be of great consequence in the regulation by glucagon. Similar conclusions have also been drawn previously by Adam & Haynes (1969).

Treatment of the cells with glucagon and adrenaline also increased the intramitochondrial concentration of ATP while having no significant effect on that of ADP, thus producing an elevated [ATP]/[ADP] ratio within the mitochondrial matrix, confirming previous work (Adam & Haynes, 1969; Halestrap, 1978; Titheradge *et al.*, 1979). Challenging the cells with insulin did not significantly alter either the mitochondrial concentrations of ATP or ADP; however, when added in the presence of glucagon or adrenaline, it decreased the effect of the hormones on the ATP concentration and lowered the [ATP]/[ADP] ratio in parallel with pyruvate carboxylation, thus supporting the concept that the elevated concentration of ATP and the increased [ATP]/[ADP] ratio are limiting factors in the regulation of pyruvate carboxylation in both the isolated mitochondria and the intact cell.

From the analysis of the concentration of metabolites in digitonin-treated cells, Siess *et al.* (1977) have suggested that glutamate may also be a potent regulator of pyruvate carboxylation *in vivo*. Measurement of the intramitochondrial concentration of glutamate under the conditions used in the CO_2-fixation assay gave values of less than 0.4nmol/mg of protein, corresponding to an intramitochondrial concentration of below 0.5mM. The concentration of glutamate required for half-maximal inhibition of pyruvate carboxylase is known to be greater than 5mM (Scrutton & White, 1974), and therefore it is unlikely to have any significant role in the regulation of pyruvate carboxylase activity in the isolated mitochondria with pyruvate alone as the substrate, although this may not be the case when other substrates are used, for example alanine (Martin & Titheradge, 1983).

In conclusion, the data presented indicate that the rate of pyruvate carboxylation is sensitive to acute insulin administration, but only when the activity of the enzyme has been enhanced after treatment with either glucagon or catecholamines, but not with vasopressin or angiotensin. Alterations in the flux of pyruvate through the enzyme are apparent in both the isolated mitochondria and filipin-treated cells, indicating that the effect of hormones at this site is not merely a reflection of the mitochondrial isolation procedure or the presence of a stimulated reaction distal in the pathway to that of pyruvate carboxylase, for example pyruvate kinase or fructose 1,6-bisphosphatase, but that it is a physiological response occurring *in vivo*. This would be consistent with the findings of Thomas & Halestrap (1981), which suggested that gluconeogenesis from lactate may be limited at the level of pyruvate carboxylase by the rate of pyruvate transport into the mitochondria in isolated hepatocytes. The characteristics of the mitochondrial response to both gluconeogenic hormones and insulin are in excellent agreement with the effect of the hormones on the rate of glucose synthesis and are entirely compatible with the concept that pyruvate carboxylase may occupy a key role in the regulation of gluconeogenesis from C_3 precursors.

It is suggested that the effect of hormone treatment on the enzyme as it exists in the isolated mitochondria is exerted by alterations in the concentrations of effector molecules, principally the concentrations of adenine nucleotides and the [ATP]/[ADP] ratio and the rate of pyruvate entry into the organelle (Adam & Haynes, 1969; Titheradge & Coore, 1976*a,b*; Halestrap, 1978; Martin & Titheradge, 1983), with acetyl-CoA and glutamate being of minor importance. However, this does not preclude acetyl-CoA and glutamate being important in the control of the enzyme *in vivo* and, indeed, these factors may be superimposed on the [ATP]/[ADP] ratio and supply of pyruvate and may contribute towards the quantitative differences in the magnitude of the effect of hormones in filipin-treated cells as compared with the isolated mitochondria.

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