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Action of phenylephrine on protein synthesis in liver cells

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The α-adrenergic agonist phenylephrine was found to inhibit protein labelling from [^{3}H]valine in isolated liver cells. This effect is only observable under conditions of partial Ca^{2+} depletion and in cells displaying maximal rates of protein labelling, i.e. cells isolated from fed animals or from starved animals when incubated in the presence of alanine. The ability of phenylephrine to inhibit protein labelling at near-saturating concentrations of the amino acid precursor indicates that this α-agonist actually decreases the rate of protein synthesis. The possibility that phenylephrine acts by making cellular Ca^{2+} availability further limiting can be ruled out, since alanine stimulates protein labelling under conditions of severe Ca^{2+} depletion obtained by pretreatment of the cells with EGTA. The following observations indicate that the phenylephrine action may be mediated by an increase in cellular cyclic AMP content: (1) a close relationship was found between the abilities of phenylephrine to inhibit protein labelling and to increase cyclic AMP content; (2) cyclic AMP mimics the phenylephrine action only in cells partially depleted of Ca^{2+}; (3) the α_1-antagonist prazosin, which inhibited the phenylephrine-mediated increase in cyclic AMP, also abolished the effect on protein synthesis.

INTRODUCTION

Although the liver is one of the most active organs in protein synthesis, the mechanisms underlying the regulation of this process are poorly understood. It has been suggested [1] that a mechanism similar to that described in reticulocytes, which involves the phosphorylation of one of the initiation factors [2,3], could operate in liver cells. However, this hypothesis has so far not been supported by any conclusive experimental evidence. Two lines of evidence suggest, nevertheless, that a phosphorylation process could play a role: first, during starvation, the initiation step of hepatic protein synthesis is impaired [4,5]; second, the plasma molar ratio of glucagon to insulin is increased during the transition from the fed to the starved state [6], and it has previously been reported that hepatic protein synthesis responds readily to glucagon [7,8]. This hormone is known to perturb liver metabolism through a rise in the intracellular content of cyclic AMP, which activates certain protein kinases [9–15]. Some of us previously reported that acute changes in the rate of hepatic protein synthesis *in vivo* induced by glucagon do not seem to correlate with variations in the cyclic AMP content [16,17]. Nevertheless, glucagon also has been reported to perturb Ca^{2+} fluxes in liver cells [18–21], and a role for Ca^{2+} in the regulation of protein synthesis in liver [22,23] and other eukaryotic cells [24–27] has been described. On the basis of these observations, we decided to determine if acute perturbations of Ca^{2+} homoeostasis induced by hormones, or other agents, could lead to changes in the rate of protein synthesis through the activation of Ca^{2+}-dependent protein kinases.

The work reported here elucidates the role of acute perturbations of Ca^{2+} fluxes in the control of hepatic protein synthesis. Phenylephrine, an α-adrenergic agonist, was studied because of the correlation that exists between its ability to mobilize Ca^{2+} from intracellular stores and the perturbation of metabolic functions [28–30]. Our results indicate that phenylephrine produces an acute inhibitory effect on protein synthesis in liver cells, but only under conditions of partially depleted Ca^{2+} content. Since only under these conditions is phenylephrine able to increase cyclic AMP content, it is concluded that a concerted action of these two intracellular messengers, Ca^{2+} and cyclic AMP, mediates the α-adrenergic-agonist induced acute rate changes in hepatic protein synthesis.

EXPERIMENTAL

Animals

Male Wistar albino rats, weighing 200–220 g were used. The animals were fed *ad libitum* until the experiment was started unless indicated otherwise.

Chemicals

[U-^{3}H]Valine (10 Ci/mol) was purchased from Amersham International (Amersham, Bucks., U.K.). All reagents were of the highest possible purity, and most of them were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Collagenase was purchased from Worthington (Freehold, NJ, U.S.A.).

Isolation and incubation of liver cells

The general procedure for isolation of liver cells was described previously [31]. The Ca^{2+} content of cells obtained by this standard procedure was 4.1 ± 0.28 nmol/mg dry wt., and these will be termed 'normal-Ca^{2+}-containing cells'. To obtain Ca^{2+}-depleted cells, the standard procedure was modified as follows: Krebs–Ringer bicarbonate buffer (KRB) without Ca^{2+} and filtered through a column of Chelex specifically to

Abbreviation used: KRB, Krebs–Ringer bicarbonate buffer.

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remove any Ca^{2+} contamination (KRB free of Ca^{2+}) was used to pre-perfuse the liver for 20 min. Then KRB containing 0.1 mM-$CaCl_2$ and approx. 1 mg of collagenase/ml was recirculated for about 20 min. The collagenase was removed by an additional flow-through perfusion period of 5 min with KRB free of Ca^{2+}. The cell suspensions so obtained were washed at least three times with KRB free of Ca^{2+} before their experimental use.

In some experiments, further depletion of Ca^{2+} was achieved by incubation at 37 °C for 10 min with 0.68 mM-EGTA.

The cells were incubated at 37 °C in KRB containing 2% (v/v) Ficoll 70, either in the absence of Ca^{2+} or with normal Ca^{2+} content. The pH of the incubation mixture was 7.4 after equilibration O_2/CO_2 (19:1).

Determination of protein-synthesis activity

L-[U-^{3}H]Valine was used as the amino acid precursor, since it is poorly oxidized or transaminated by the liver cells [32]. Routinely, 1 ml portions of liver cells (40–60 mg wet wt./ml) were placed in plastic 25 ml Erlenmeyer flasks and incubated at 36.5 °C in a rotary shaker for 1 h. The incubation medium contained either tracer doses of L-[U-^{3}H]valine or 0.5 mM-L-[U-^{3}H] valine (sp. radioactivity 10 Ci/mol). The latter concentration seems to be close to saturation [31]; thus minor changes in the size of the valine pool utilized for protein synthesis would not induce appreciable changes in the rate of protein labelling. Furthermore, the rate of valine production by the cells, determined by a procedure previously described [33], was 0.35 μmol/g wet wt. when calculated for the 30–60 min interval of the incubation. The presence of phenylephrine in the incubation medium did not alter this rate significantly. When indicated, phenylephrine was added after 5 min of incubation. At 0, 30 and 60 min triplicate samples (0.1 ml) were taken and immediately added to 10% (v/v) trichloroacetic acid and processed as described previously [31]. The rate of protein labelling was calculated for the 30–60 min interval, i.e. when the incorporation of radioactivity into proteins was linear. From these data, and taking into account that the counting efficiency was 9%, the fractional rate of protein synthesis was calculated to be 11.7%. This value is similar to that reported by Seglen & Solheim [34], and is 16% of the fractional rate of hepatic protein synthesis *in vivo* [8]. The fractional rate in hepatocytes obtained from 48 h-starved animals was calculated to be 4.2%, i.e. 30% of the fractional rate determined in hepatocytes obtained from fed animals.

Determination of cyclic AMP content

Portions of the cell suspension (0.8 ml; 50–60 mg/ml) were transferred to tubes containing 200 μl of 30% (w/v) $HClO_4$. The tubes were centrifuged at 13000 rev./min for 2 min and the supernatants neutralized with 10 M-KOH. The cyclic AMP content was determined by the procedure described by Steiner *et al.* [35].

Determination of the rates of glycogenolysis and gluconeogenesis

For determination of the rate of glycogenolysis, hepatocytes from fed rats were utilized. For determination of the rate of gluconeogenesis from 10 mM-alanine, hepatocytes from 48 h-starved rats were used. Owing to the acid-lability of Ficoll 70, the incubation medium was supplemented with 1.5% (w/v) gelatin. After incubation at 37 °C for 0, 30 and 60 min, portions of the cell suspension were processed as described previously [31] for the enzymic determination of glucose.

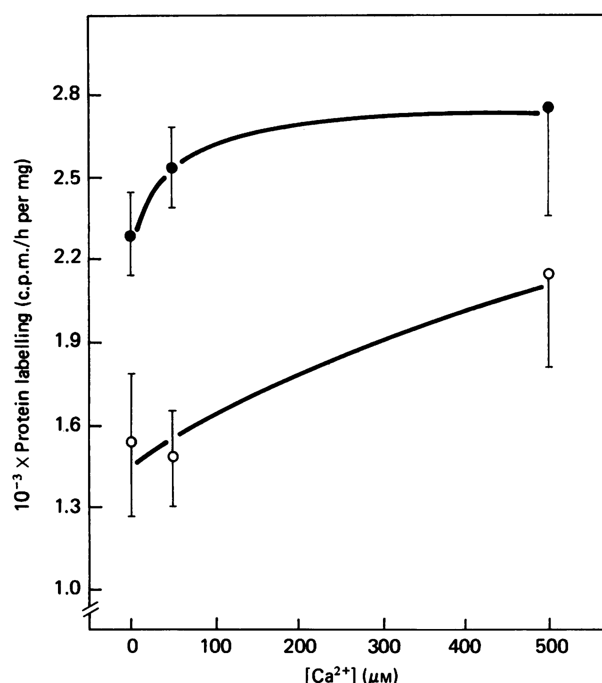

Fig. 1. Effect of phenylephrine on the rate of protein labelling in isolated hepatocytes, partially depleted of Ca^{2+}, as a function of extracellular Ca^{2+} concentration

Hepatocytes partially depleted of Ca^{2+} were obtained from rats fed *ad libitum* as described in the Experimental section. Rates of protein labelling were determined by using tracer doses of [^{3}H]valine and in the absence (●) and in the presence (○) of phenylephrine (20 μM). The points represent mean values of five cell preparations, each run in triplicate, and the vertical bars give the S.E.M. The differences between the mean values obtained in the absence and in the presence of phenylephrine were statistically significant (by paired t test; $P < 0.05$) at any concentration of Ca^{2+} in the extracellular medium.

Determination of Ca^{2+} content

For this determination, 800 μl of cell suspension (50–60 mg/ml) was placed in Eppendorf tubes on a layer of a mixture of silicone oil (Siliconas Hispania, S.A.; $d = 1.075$) and olive oil (4:1, v/v). The tubes were immediately centrifuged at 13000 rev./min for 2 min. After careful removal of the oil layer, the pellet was resuspended in 0.2 ml of 14% $HClO_4$. The samples were then diluted with 800 μl of 0.1% $LaCl_3$. The Ca^{2+} content was determined by atomic absorption spectrometry.

RESULTS

Cytosolic free Ca^{2+} concentration is a function of the total Ca^{2+} content, which ultimately is correlated with the extracellular Ca^{2+} concentration [36]. We began by studying the effect of Ca^{2+} mobilization from intracellular stores, over a wide range of cytosolic free Ca^{2+} concentrations. This was done by determining the effect

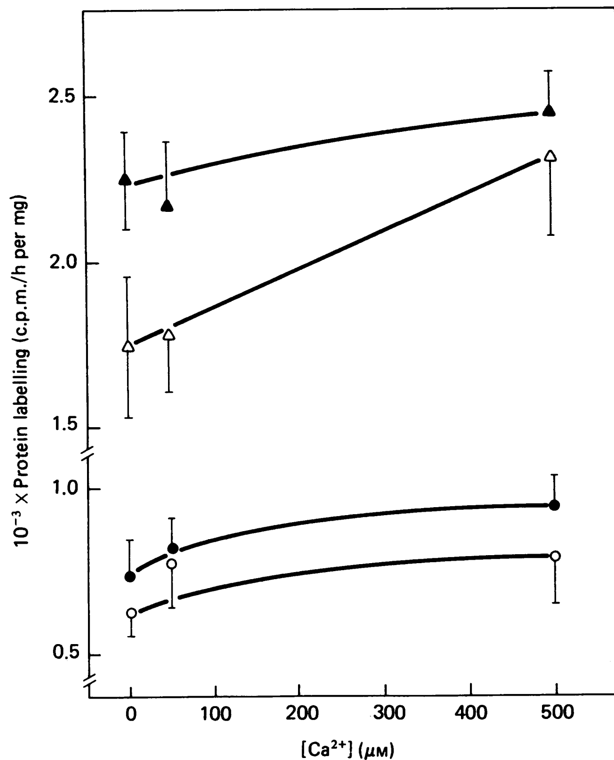

Fig. 2. Effect of phenylephrine on the rate of protein labelling in hepatocytes partially depleted of Ca^{2+} obtained from starved rats, as a function of extracellular Ca^{2+} concentration

Hepatocytes partially depleted of Ca^{2+} were obtained from rats starved for 48 h. Rates of protein labelling from tracer doses of [^{3}H]valine were determined in the absence (●, ○) and in the presence (▲, △) of 10 mM-alanine and in the absence (●, ▲) and in the presence (○, △) of phenylephrine (20 μM). The points are mean values of six cell preparations, each run in triplicate, and the vertical bars give the S.E.M.. The differences between the mean value obtained in the control and in the presence of phenylephrine and alanine and in the absence of Ca^{2+} were statistically significant (by paired t test: $P < 0.05$).

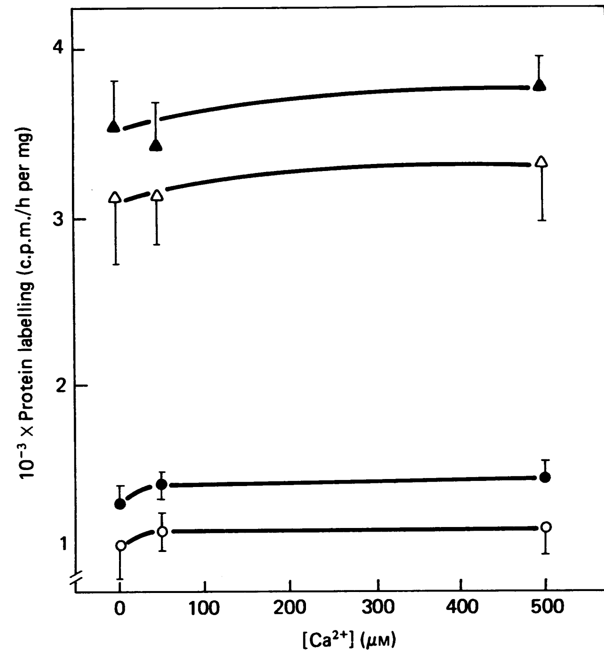

Fig. 3. Effect of phenylephrine on the rate of protein labelling in hepatocytes, isolated from starved rats, as a function of the extracellular Ca^{2+} concentration

Hepatocytes with a standard Ca^{2+} content, obtained from 48 h-starved rats, were used in these experiments. The rates of protein labelling from tracer amounts of [^{3}H]-valine were determined as described in the Experimental section. The incubation medium contained no substrates (●, ○) or 10 mM-alanine (▲, △) in the presence (○, △) or in the absence (●, ▲) of phenylephrine (20 μM) and the indicated Ca^{2+} concentration. The points are mean values of five cell preparations, each run in triplicate, and the vertical bars give the S.E.M. The differences between the mean values for control and phenylephrine-treated were not statistically significant under any condition.

of phenylephrine on liver cells, partially depleted of Ca^{2+}, and incubated in the presence of different amounts of extracellular Ca^{2+}. Depletion of Ca^{2+} by incubation with EGTA was found to cause deleterious effects; the cells became unresponsive to hormonal agents (results not shown). The data in Fig. 1 were obtained in cells only partially depleted of Ca^{2+}, obtained from fed animals which retained their hormone-responsiveness intact. As shown in Fig. 1, replenishment of Ca^{2+} stores by incubating the cells with increasing concentrations of Ca^{2+} enhanced protein labelling from [^{3}H]valine, in agreement with previous reports [22]. The presence of phenylephrine (20 μM) in the incubation medium resulted in a statistically significant decrease in the rate of protein labelling. The lower the Ca^{2+} availability, the more pronounced was the observed inhibition.

Influence of the nutritional status of the animals on the response of isolated cells to phenylephrine

The rate of hepatic protein synthesis *in vivo*, or in isolated hepatocytes, is inhibited during starvation [4,5], whereas the rate of proteolysis is stimulated [37]. Both parameters are sensitive to the availability of certain substrates in the starved animal. Alanine, in particular, seems to be highly effective in restoring rates of protein synthesis, either *in vivo* or in isolated liver cells, to values observed in the normal fed animal [5]. When phenylephrine was tested in hepatocytes from starved animals (Fig. 2), in the absence of any substrate, no detectable decrease in protein labelling was observed at all concentrations of Ca^{2+} studied. The presence of alanine restored rates of protein labelling to values similar to those previously shown in cells from fed animals (Fig. 1). Phenylephrine was able to prevent partially the stimulatory effect of alanine, but only in the absence of extracellular Ca^{2+} (Fig. 2). These results indicate that phenylephrine does not perturb basal rates, but acts only on the mechanism(s) which allows maximal, stimulated, rates of protein synthesis under conditions where the availability of extracellular Ca^{2+} is limiting. The latter requirement is further emphasized by results presented in Fig. 3; when 'normal' non-Ca^{2+}-depleted hepatocytes (isolated from starved rats) were used, phenylephrine had no detectable effect in decreasing protein labelling, regardless of the extracellular Ca^{2+} concentration. The phenylephrine dose/response curve presented in Fig. 4

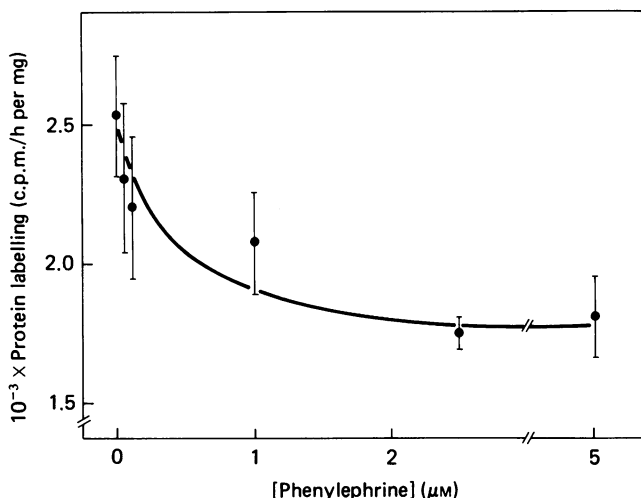

Fig. 4. Dose/response of the phenylephrine effect on the rate of protein labelling in liver cells

Hepatocytes partially depleted of Ca^{2+} were obtained from rats starved for 48 h. Rates of protein labelling from tracer amounts of [^{3}H]valine were determined by incubating the cells in Ca^{2+}-free buffers in the presence of 10 mM-alanine and the indicated concentrations of phenylephrine. The points represent mean values of four cell preparations, each run in triplicate, and the vertical bars give the S.E.M. The effects of phenylephrine concentrations above 0.1 μM were statistically significant (by paired t test: $P < 0.05$).

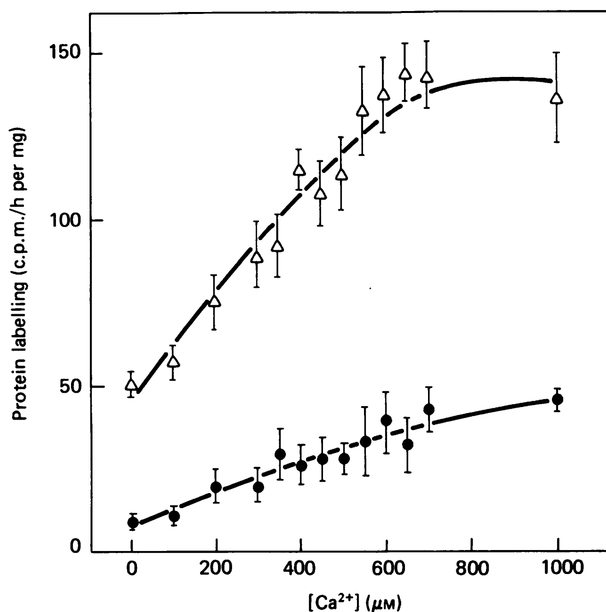

Fig. 5. Effect of extracellular Ca^{2+} concentration on the rate of protein synthesis in hepatocytes depleted of Ca^{2+}, obtained from starved rats

Hepatocytes, obtained from 48 h-starved rats, were depleted of Ca^{2+} as described in the Experimental section. The rates of protein synthesis were determined by the incorporation of radioactivity into proteins from 0.5 mM-[^{3}H]valine (sp. radioactivity 10 Ci/mol). The cells were incubated in a buffer containing 0.68 mM-EGTA and the indicated concentrations of Ca^{2+} without (●) or with (△) 10 mM-alanine. The points represent mean values of four experiments, and the vertical bars give the S.E.M.

Table 1. Effect of α- and β-antagonists on the phenylephrine-induced inhibition of protein labelling and increase in cyclic AMP content in cells partially depleted of Ca^{2+}

Hepatocytes obtained from 48 h-starved rats were partially depleted of Ca^{2+} as described in the Experimental section. The incubation medium contained 0.5 mM-[^{3}H]valine (sp. radioactivity 10 Ci/mol) and 10 mM-alanine. Values are means of at least three preparations, each run in triplicate, ±S.E.M. By t test: $*P < 0.02$; $**P < 0.05$.

Additions	Concn. (μM)	Protein labelling (c.p.m./h per mg wet wt.)	Cyclic AMP content (nmol/mg wet wt.)
None (control)	–	1191±133	0.25±0.02
Phenylephrine	50	813±30*	0.53±0.1**
Phenylephrine	50		
+Propranolol	1	773±42**	0.52±0.02**
+Prazosin	1	1034±112	0.34±0.06
+Yohimbine	1	846±91	0.47±0.1

shows that the concentration required to obtain maximal inhibition of protein labelling (2–5 μM) is similar to that reported for other metabolic actions of this α-adrenergic agent [38].

The transient increase in cytosolic free Ca^{2+} induced by α-adrenergic agents, secondary to Ca^{2+} mobilization from intracellular stores [28,30], is accompanied by cellular Ca^{2+} efflux, leading to net Ca^{2+} losses when the agonist is present [39]. Hence the possibility that the observed effect of phenylephrine in preventing alanine stimulation of protein labelling was caused by a cellular Ca^{2+} depletion, rather than interacting with the protein-synthesis machinery, was considered. The data presented in Fig. 5 (from experiments carried out with cells severely depleted of Ca^{2+} by pretreatment with EGTA, and incubated in the presence of increasing concentrations of extracellular Ca^{2+}) indicates that the alanine response was observable over a wide range of Ca^{2+}-loading conditions. Furthermore, the alanine response was even quantitatively greater when the cells were not supplemented with Ca^{2+}.

Effects of phenylephrine and Ca^{2+} on rates of protein synthesis

If protein synthesis and proteolysis share the same amino acid pool [40,41], then the action of phenylephrine in decreasing protein labelling described above might be the result of an increased rate of proteolysis. This possibility can be disregarded on the basis of data presented in Table 1. As shown, phenylephrine was similarly effective at near-saturating concentrations of the amino acid precursor (0.5 mM) when a dilution effect, secondary to an increased rate of proteolysis, would cause only negligible effects on the rate of protein labelling [31].

Role of cyclic AMP in the phenylephrine-induced inhibition of protein labelling

The effect of phenylephrine in decreasing protein labelling, observed only in partially depleted cells, does not support the idea that its effect was mediated by an

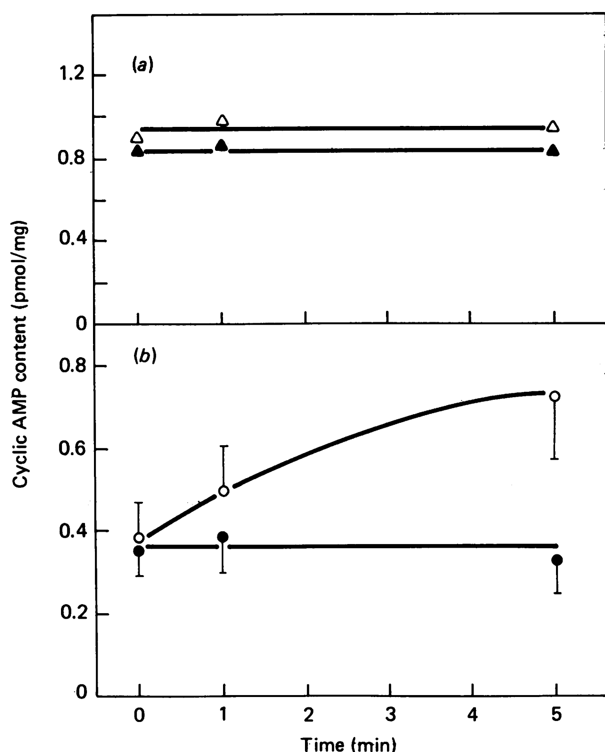

Fig. 6. Effect of phenylephrine and Ca^{2+} on the cyclic AMP content of isolated hepatocytes

Hepatocytes, obtained from fed rats, were partially depleted of Ca^{2+} as described in the Experimental section. The cells were resuspended in Hanks medium with (*a*) or without (*b*) 1.3 mM-Ca^{2+}. When indicated, phenylephrine (△, ○) was added to give a final concentration of 50 μM. Cyclic AMP content was determined as described in the Experimental section. The points represent mean values of three cell preparations, run in triplicate, and the vertical bars give the S.E.M. The differences in cyclic AMP content at zero time between Ca^{2+}-depleted and Ca^{2+}-restored cells and at 5 min in Ca^{2+}-depleted cells with and without phenylephrine were statistically significant (by *t* test; $P < 0.05$).

increase in cytosolic free Ca^{2+}. Fig. 6 shows that phenylephrine was able to increase cyclic AMP only in Ca^{2+}-depleted cells, in agreement with a previous report [42]. This suggests a role for cyclic AMP in the phenylephrine-induced inhibition of protein synthesis. The results in Table 1 show the relationship between the ability of phenylephrine to decrease protein labelling and the rise in cyclic AMP content. Prazosin was the only adrenergic antagonist studied (Table 1) able to prevent both phenylephrine effects, i.e. the decrease in protein labelling and the rise in cellular cyclic AMP content. This indicates that its action is exerted through the α_1-receptors.

The possible participation of cyclic AMP in the α-adrenergic response at low Ca^{2+} concentration seems to be supported by the data in Table 2. This shows that exogenously added cyclic AMP is effective in decreasing protein labelling in isolated hepatocytes, but only in Ca^{2+}-depleted cells incubated in the absence of extracellular Ca^{2+}.

Table 2. Effect of cyclic AMP on rates of protein labelling in liver cells

Hepatocytes obtained from fed rats were partially depleted of Ca^{2+} as described in the Experimental section. The incubation medium contained tracer amounts of [^{3}H]-valine and 50 μM-cyclic AMP. Rates of protein labelling were determined as described in the Experimental section. The data are means of four cell preparations, each run in triplicate, ±S.E.M. By paired *t* test; *$P < 0.02$.

	Ca^{2+} (mM)	Protein labelling (c.p.m./h per mg wet wt.)	Inhibition (%)
Control	0	1573±112	–
+Cyclic AMP	0	984±103*	38
Control	1.3	1963±251	–
+Cyclic AMP	1.3	1899±201	4

DISCUSSION

The rate of hepatic protein synthesis decreases (Figs. 1 and 2) in the nutritional transition from the fed to the starved state. Phenylephrine was found not to perturb protein synthesis in hepatocytes isolated from starved animals when incubated in the absence of substrates (Fig. 3). A similar observation has been reported with glucagon, which can acutely induce a 40% decrease in the rate of hepatic protein synthesis in fed animals, but is ineffective in starved animals [43]. In rat liver, the lowest rate of protein synthesis appears to be that observed in starved animals. This is in contrast with other mammalian cell systems, in which protein synthesis can be effectively switched off [44]. Apparently, this rate is the minimum allowed to spare amino acids for a most vital function, such as gluconeogenesis [17], without compromising cellular functional viability. Long-term adaptations, or a limited supply of amino acids or other substrates, do not seem to be responsible for the lack of phenylephrine action in cells from starved rats. This follows from the observation that alanine alone acutely increases the rate of protein labelling, rendering the liver cells responsive to this agonist (Fig. 3).

The finding that phenylephrine inhibited protein labelling confirms and extends previous reports by other authors [23,45]. In liver cells, the action of phenylephrine was only observed under conditions of partial Ca^{2+} depletion (Figs. 1–3). Two possibilities can be considered to explain this: first, its effect could be the consequence of an increase in cytosolic free Ca^{2+}, secondary to its mobilization from intracellular stores [28,30]; second, the phenylephrine induced cellular Ca^{2+} efflux could make the availability of this ion limiting. A Ca^{2+} requirement for protein synthesis has been reported in several mammalian cells [22–27]. However, as the data on isolated hepatocytes show (Fig. 5), a dependency of protein synthesis on the extracellular Ca^{2+} concentration was only observed in cells severely depleted of Ca^{2+} by pretreatment with EGTA. Under these conditions, both basal and stimulated rates of protein synthesis show similar dependencies of Ca^{2+}. However, phenylephrine is effective only in the presence of alanine. This finding does not support the idea that cellular Ca^{2+} depletion could mediate phenylephrine action.

In cells less severely depleted of calcium, the response of increased protein labelling as the extracellular Ca^{2+} was increased was considerably less (Figs. 1–3). The total Ca^{2+} content of Ca^{2+}-depleted and not-depleted cells was 0.35 ± 0.02 and 4.1 ± 0.28 nmol/mg dry wt. respectively. The free cytosolic Ca^{2+} is a function of the total cellular content [36]. Thus the observed lack of correlation between cellular Ca^{2+} content and rates of protein synthesis does not support the idea that variations in the concentration of cytosolic free Ca^{2+} by itself plays a major role in the regulation of bulk protein synthesis. On these grounds, it does not seem probable that the action of phenylephrine in decreasing protein labelling could be the result of a perturbation of cellular Ca^{2+} homoeostasis.

The inhibition of protein labelling is not the only action of phenylephrine which occurs in the absence of extracellular Ca^{2+}. It has been reported that this α-agonist causes an activation of a protein kinase(s) and histone kinase and inactivates pyruvate kinase [46], and elevated cyclic AMP content [42] only in liver cells depleted of calcium. The latter observation prompted us to consider the possibility that this cyclic nucleotide could mediate the inhibitory action on protein synthesis. Two observations seems to support this possibility: first, the correlation betewen the ability of phenylephrine to inhibit protein labelling and to elevate the cellular content of cyclic AMP (Fig. 6 and Table 1) occurs only in Ca^{2+}-depleted cells; the second is the fact that exogenously added cyclic AMP inhibited protein labelling only under similar conditions (Table 2). A lack of effect of cyclic AMP on protein synthesis at physiological Ca^{2+} concentrations is in agreement with previous reports [47].

Apparently, the concerted action of low Ca^{2+} concentration and an increase in cyclic AMP seems to mediate the phenylephrine action in inhibiting protein labelling. Low Ca^{2+} may act by making proteins accessible to cyclic AMP through conformational changes. It should be noted that a concerted action of both Ca^{2+} and cyclic AMP has been postulated to occur in the action of Ca^{2+}-mobilizing hormones [48–50]. The observation that Ca^{2+} is able to increase cyclic AMP by itself (Fig. 6) supports this proposal.

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