Comparison of the effects of [leucine]enkephalin and angiotensin on hepatic carbohydrate and cyclic nucleotide metabolism

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The effects of [leucine]enkephalin and angiotensin on hepatic carbohydrate and cyclic nucleotide metabolism are compared. Both peptides stimulated glycogenolysis as a result of an increase in phosphorylase *a* activity and enhanced glucose synthesis from [2-¹⁴C]pyruvate, although neither had any significant effect on pyruvate kinase activity. Although the magnitudes of the effects of both peptides on glycogenolysis were comparable and unaffected by the presence of insulin, [Leu]enkephalin proved to be more efficacious in enhancing gluconeogenesis, the response being comparable with that to glucagon. Both effectors decreased the intracellular concentration of cyclic AMP in hepatocytes when incubated under control conditions and after addition of sub-optimal concentrations of glucagon. This was correlated with the ability of the two peptides to inhibit both basal and hormone-stimulated adenylate cyclase activity in purified liver plasma membranes.

INTRODUCTION

Previous studies have demonstrated that addition of the opioid peptides [Leu]enkephalin, [Met]enkephalin, dynorphin-(1–13) and β -endorphin to isolated hepatocytes results in rapid dose-dependent stimulation of both glycogenolysis and gluconeogenesis (Allan *et al.*, 1983; Matsumura *et al.*, 1984; Leach *et al.*, 1985; Leach & Titheradge, 1986). For β -endorphin, this has been attributed to an increase in cyclic AMP within the cells (Matsumura *et al.*, 1984), whereas the effect of the enkephalins and dynorphin-(1–13) is via a cyclic-nucleotide-independent mechanism, involving the turnover of inositol lipids and a mobilization of intracellular Ca^{2+} (Leach & Titheradge, 1986; Leach *et al.*, 1986). It is evident from the literature that the effects of hormones acting through changes in intracellular Ca^{2+} are not necessarily equivalent (Blackmore *et al.*, 1979; Dehaye *et al.*, 1981; Jard *et al.*, 1981; Cardenas-Tanus *et al.*, 1982; Binet *et al.*, 1985; Charest *et al.*, 1985; Lynch *et al.*, 1985; Mine *et al.*, 1986; Kleineke & Söling, 1987). Measurements of glucose synthesis from [2-¹⁴C]pyruvate (Allan *et al.*, 1983) and inositol lipid turnover (Leach *et al.*, 1986) have suggested that the effects of the enkephalins are significantly different from those of the catecholamines, and in the present study we have compared the effects of [Leu]enkephalin with those of angiotensin on a number of aspects of carbohydrate metabolism. We have also compared the effects of the opioid peptide with those of angiotensin on cyclic nucleotide metabolism, as preliminary studies indicated that, in addition to mobilizing intracellular Ca^{2+} , the enkephalins suppressed the formation of cyclic AMP in the liver (Leach & Titheradge, 1986). Although this is a characteristic of both α_2 -adrenergic agonists and the vasoactive peptides angiotensin and vasopressin, it is also suggestive of the involvement of discrete opiate receptors in liver, as it is well established that opiates can inhibit adenylate cyclase activity in neuronal tissue, particularly striatal membranes and the neuroblastoma $\times$ glioma hybrid cell line NG108-15 (Sharma *et al.*, 1975; Blume *et al.*, 1979; Cooper *et al.*, 1982).

EXPERIMENTAL

Materials

[Leu]enkephalin, phenylephrine, angiotensin, insulin and collagenase were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). [U-¹⁴C]Glucose 1-phosphate and [α -³²P]ATP were obtained from Amersham International (Amersham, Bucks., U.K.). Glucagon was a gift from Novo Laboratories (Basingstoke, Hants., U.K.). All other chemicals either were of AnalaR grade from BDH Chemicals (Poole, Dorset, U.K.) or were from Sigma.

Methods

Hepatocytes were prepared from fed male Sprague-Dawley rats weighing 180–240 g, essentially by the method of Berry & Friend (1969) as described previously (Allan *et al.*, 1983). The cells were resuspended in Krebs–Henseleit (1932) bicarbonate buffer, pH 7.4, to a final concentration of approx. 60 mg wet wt./ml, and incubated at 37 °C under an atmosphere of O_2/CO_2 (19:1). The cells were routinely preincubated for 30 min before the addition of the hormones or effectors and, unless stated otherwise, samples were removed after a further 2 min of incubation for the determination of phosphorylase *a* or cyclic AMP. It has previously been demonstrated that preincubation of the cells with insulin is more effective in antagonizing the effects of both glucagon and catecholamines than is adding the two effectors together (Massague & Guinovart, 1978; Dehaye *et al.*, 1981; Thomas & Williamson, 1983). Preliminary experiments in our laboratory confirmed this observation and indicated that a 5 min preincubation period before the addition of glucagon produced an optimal response. Hormones and effectors were added as 100-fold concentrates in 0.9% NaCl containing 1 mg of bovine serum albumin/ml; control incubations received an equivalent volume of vehicle. Glycogenolysis and glucose synthesis from [2-¹⁴C]pyruvate were measured over a 15 min incubation period. Rat liver plasma membranes were prepared essentially by the method of Pohl (1976) as modified by Campanile *et al.* (1982) for

Table 1. Effect of [Leu]enkephalin, glucagon and angiotensin on phosphorylase activity, glycogenolysis, glucose synthesis and pyruvate kinase activity in isolated hepatocytes

After a 30 min preincubation period, [Leu]enkephalin, angiotensin or glucagon was added to the cells, to final concentrations of 10 μ M, 1 μ M and 1 nM respectively. Controls received the appropriate vehicle. Glycogenolysis and phosphorylase *a* activity were measured in cells incubated in the absence of substrates. For the determination of pyruvate kinase activity and glucose synthesis from [2-¹⁴C]pyruvate, the substrate was added simultaneously with the effectors at the end of the preincubation period. Samples were removed after 4 and 5 min for the determination of pyruvate kinase and phosphorylase *a* activity respectively. Glycogenolysis and glucose synthesis were measured over 15 min as described in the Experimental section. Pyruvate kinase activity is expressed as the ratio of the activity measured at 0.5 mM-phosphoenolpyruvate to that at 5.0 mM-phosphoenolpyruvate plus 20 μ M-fructose 1,6-bisphosphate. Results are expressed as means $\pm$ S.E.M., with the numbers of different cell preparations given in parentheses: **P* < 0.05; ***P* < 0.01.

Addition	Activity (μ mol/min per g dry wt.)			Pyruvate kinase activity ratio
	Glycogenolysis	Phosphorylase <i>a</i>	Gluconeogenesis	
Control	3.45 $\pm$ 0.44 (8)	41.2 $\pm$ 4.2 (8)	1.34 $\pm$ 0.13 (6)	0.32 $\pm$ 0.02 (10)
Glucagon	7.62 $\pm$ 1.06 (8)**	89.5 $\pm$ 7.2 (8)**	2.22 $\pm$ 0.17 (6)**	0.12 $\pm$ 0.02 (10)*
Angiotensin	7.21 $\pm$ 0.59 (8)**	81.7 $\pm$ 5.8 (8)**	1.83 $\pm$ 0.12 (6)**	0.29 $\pm$ 0.02 (10)
[Leu]enkephalin	7.46 $\pm$ 0.70 (8)**	84.6 $\pm$ 5.2 (8)**	2.21 $\pm$ 0.17 (6)**	0.27 $\pm$ 0.02 (10)

the preservation of inhibitory effects of hormones on adenylate cyclase activity. The preparation medium consisted of 10 mM-Tris, pH 7.4, 5 mM-Na₂EDTA and 0.1% β -mercaptoethanol.

Phosphorylase *a* activity was determined as described previously (Leach *et al.*, 1985). Rates of glycogenolysis were determined in cells incubated in the absence of substrates by the output of glucose. Glucose output was measured in trichloroacetic acid extracts (final concn. 10%, w/v) by using glucose oxidase (Bergmeyer & Bernt, 1974). Glucose synthesis from [2-¹⁴C]pyruvate was measured as described previously (Allan *et al.*, 1983). The substrate used was a mixture of [2-¹⁴C]pyruvate (sp. radioactivity 50 mCi/mmol) plus unlabelled lactate in a ratio of 1:4 to give a final substrate concentration of 5 mM. Pyruvate kinase activity was measured as described by Garrison *et al.* (1979). After incubating the cells with effectors for 4 min, 3 ml of cells was added to an equal volume of buffer containing 100 mM-NaF, 10 mM-EDTA and 10 mM-Tes, pH 7.5, and the cells were immediately frozen in liquid N₂. After thawing on ice, the enzyme was extracted by precipitation with an equal volume of 3.9 M-(NH₄)₂SO₄ and resuspended in buffer containing 50 mM-NaF, 10 mM-Tes, pH 7.5, and 20% (v/v) glycerol. Cyclic AMP was determined in neutralized HClO₄ extracts of cells by radioimmunoassay as described by Brooker *et al.* (1979). Adenylate cyclase activity was measured by the method of Crane *et al.* (1982). The assay medium consisted of 50 mM-Tris, pH 8.0, 0.5 mM-[α -³²P]ATP (1 $\times$ 10⁶ c.p.m./tube), 10 mM-MgCl₂, 1 mM-EDTA, 0.1 mg of bovine serum albumin/ml, 0.4 mM-3-isobutyl-1-methylxanthine, 20 units of creatine kinase/ml, 10 mM-phosphocreatine, 5 units of adenosine deaminase/ml, 0.1 mM-GTP and 100 mM-NaCl. Hormones and peptides were added as indicated. Cyclic [³²P]AMP formed in the reaction was separated from [³²P]ATP by column chromatography on Dowex AG 50W-X4 and alumina as described by Salomon (1979). The recovery was determined by the inclusion of cyclic [³H]AMP (Salomon, 1979) and averaged 82%. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V) as the standard.

Results are expressed as means $\pm$ S.E.M., with the numbers of observations given in parentheses. Statistical analysis of the results was performed by using a pooled *t* test.

RESULTS AND DISCUSSION

Comparison of the effects of [Leu]enkephalin, glucagon and angiotensin on hepatic carbohydrate metabolism

Table 1 shows the effect of addition of 10 μ M-[Leu]enkephalin to isolated hepatocytes on glycogen breakdown, phosphorylase *a* activity, glucose synthesis from [2-¹⁴C]pyruvate and pyruvate kinase activity. The effects were compared with those of angiotensin, glucagon being added as a positive control. The times of sampling and concentrations of effectors used were chosen such that a maximal response was observed with each effector on all of the parameters measured (Blair *et al.*, 1976; Chan & Exton, 1978; Hue *et al.*, 1978; Claus *et al.*, 1979; Garrison & Borland, 1979; Allan *et al.*, 1983; Leach *et al.*, 1985; Leach & Titheradge, 1986). Table 1 shows that [Leu]enkephalin rapidly increased phosphorylase *a* activity and glycogenolysis, as measured by glucose output over 15 min. Similarly, addition of the opioid peptide to the cells significantly enhanced the rate of glucose formation from a mixture of [2-¹⁴C]pyruvate plus lactate. In agreement with our previous studies (Allan *et al.*, 1983; Leach *et al.*, 1985; Leach & Titheradge, 1986), the effect of the opioid peptide was not significantly different from that of glucagon (*P* > 0.05) on any of the parameters. Although the effects of both [Leu]enkephalin and angiotensin on glycogen metabolism were comparable, the opioid peptide enhanced the rate of glucose formation to a significantly greater extent than that of angiotensin (*P* < 0.05). Despite the fact that both effectors were added at maximal concentrations, there was a 68% increase in glucose formation in the presence of [Leu]enkephalin and a 40% increase in the presence of angiotensin. The difference in magnitude of the response of gluconeogenesis to angiotensin and glucagon has been previously noted and correlated with the differential effect of the two hormones at the level of

pyruvate kinase (Chan & Exton, 1978; Garrison & Borland, 1979). Wong *et al.* (1978) have presented evidence to suggest that opiates can act at this site and that the increase in gluconeogenesis in rats after chronic morphine treatment is the result of a decrease in total pyruvate kinase activity in the liver. Therefore we have compared the ability of both angiotensin and [Leu]-enkephalin to inhibit pyruvate kinase activity measured in cell extracts, to determine whether changes in the activity of the extracted enzyme can completely account for the stimulation of gluconeogenesis. There was no effect of any of the peptides on maximal pyruvate kinase activity measured in the presence of 5 mM-phosphoenolpyruvate and 20 μ M-fructose 1,6-bisphosphate, the activities being 0.159 ± 0.013 , 0.158 ± 0.014 , 0.165 ± 0.016 and 0.151 ± 0.016 μ mol/min per mg dry wt. ($n = 10$) for control cells and cells treated with glucagon, angiotensin and [Leu]enkephalin respectively. Addition of 1 nM-glucagon significantly lowered the activity ratio of the pyruvate kinase ($P < 0.05$), the velocity measured at 0.5 mM-phosphoenolpyruvate being 40% of that in the control extracts (Table 1). This agrees favourably with previous studies (Blair *et al.*, 1976; Chan & Exton, 1978; Claus *et al.*, 1979; Garrison *et al.*, 1979). Challenging the cells with either of the Ca^{2+} -dependent agonists produced only a small decrease in the activity of pyruvate kinase measured at 0.5 mM-phosphoenolpyruvate, the mean inhibition being 7% and 15% for angiotensin and [Leu]enkephalin respectively. Although this inhibition was qualitatively the same as the effects of the two agents on glucose synthesis, neither response was significantly different from the control ($P > 0.05$). These results compare favourably with other reports, which indicate either no change (Hue *et al.*, 1978) or only a small change (Chan & Exton, 1978; Claus *et al.*, 1979) in pyruvate kinase activity after treatment of cells with hormones acting via a rise in intracellular Ca^{2+} . However, they are in contrast with the findings of Garrison *et al.*

(1979), which indicated a significant decrease in activity after treatment of cells with angiotensin or vasopressin, measured under similar conditions. From the present study, it would appear unlikely that the differential effect between the opioid peptide and angiotensin on glucose synthesis in cells prepared from the fed animal residues at the level of pyruvate kinase; however, this cannot be precluded. It is evident that the changes in the measured activity of the isolated enzyme may not reflect alterations in pyruvate kinase flux in the intact cell, which are subject to alterations in the cytosolic concentrations of allosteric effectors (Rognstad & Katz, 1977). There is some evidence to suggest that this may be the case when comparing the effects of [Leu]enkephalin and angiotensin, as we have previously demonstrated that these agents have a differential effect on lactate output by the cells. Addition of the opioid peptide decreases lactate output in hepatocytes prepared from fed animals in a similar manner to that of glucagon (Leach *et al.*, 1985), whereas addition of angiotensin either has no effect (Leach *et al.*, 1985) or stimulates lactate output (Hems *et al.*, 1978; Hue *et al.*, 1981a,b).

Effect of insulin on the increase in phosphorylase *a* by [Leu]enkephalin

Although it has been clearly established that prior incubation of hepatocytes with insulin antagonizes the effects of sub-optimal concentrations of glucagon and catecholamines on gluconeogenesis and glycogenolysis, several reports indicate that it is unable to decrease the effects of either angiotensin or vasopressin on these parameters (Hue *et al.*, 1978; Massague & Guinovart, 1978; Whitton *et al.*, 1978; Blackmore *et al.*, 1979; Strickland *et al.*, 1980). As a role for opioid peptides in the deterioration of metabolic control in diabetes has been postulated (Ipp *et al.*, 1982), it was pertinent to determine whether these direct effects of the opioid peptides could be counteracted by the presence of insulin.

Fig. 1. Effect of insulin on the activation of phosphorylase by glucagon and [Leu]enkephalin

After a 25 min preincubation period, 50 nM-insulin (●) or vehicle (■) was added to the cells, followed 5 min later by either glucagon or [Leu]enkephalin to the concentrations indicated. Samples were removed for the determination of phosphorylase *a* activity 5 min after the addition of the effector. Results are expressed as means $\pm$ S.E.M. for six different cell preparations.

Table 2. Effect of [Leu]enkephalin, glucagon and angiotensin on cyclic AMP formation and phosphorylase activity in isolated hepatocytes

After a 30 min preincubation period, effectors were added to the cells at final concentrations of 10 μM , 1 μM and 1 nM respectively for [Leu]enkephalin, angiotensin and glucagon. Samples were removed after 2 min for the determination of cyclic AMP and phosphorylase *a* as described in the Experimental section. Results are expressed as means $\pm$ S.E.M. for eight different cell preparations: ** $P < 0.01$.

Addition	Cyclic AMP (pmol/g dry wt.)	Phosphorylase <i>a</i> ($\mu\text{mol/min per g dry wt.}$)
Control	954 $\pm$ 59	27.1 $\pm$ 3.8
[Leu]enkephalin	783 $\pm$ 37**	71.0 $\pm$ 4.9**
Angiotensin	771 $\pm$ 91**	70.6 $\pm$ 5.3**
Glucagon	18 295 $\pm$ 3834**	74.6 $\pm$ 4.4**
[Leu]enkephalin + glucagon	7450 $\pm$ 2005**	71.7 $\pm$ 5.4**
Angiotensin + glucagon	9039 $\pm$ 1530**	80.0 $\pm$ 3.1**
Angiotensin + [Leu]enkephalin	725 $\pm$ 75**	83.6 $\pm$ 4.0**

Fig. 1 shows the effect of addition of 50 nM-insulin on the activation of phosphorylase by different concentrations of [Leu]enkephalin. The effect of insulin on the increase in phosphorylase *a* by glucagon was included as a positive control. Glucagon stimulated phosphorylase *a* activity over the concentration range 0.01–1 nM. Inclusion of 50 nM-insulin alone had no significant effect on the activity of phosphorylase *a* in control cells ($P > 0.05$), the basal activities being 28.6 ± 2.6 and $28.1 \pm 2.6 \mu\text{mol/min per g dry wt.}$ for control and insulin-treated cells respectively ($n = 15$). However, prior treatment of the cells with insulin significantly decreased the ability of glucagon to inhibit pyruvate kinase activity at sub-optimal concentrations ($P < 0.05$), the stimulation at 0.1 nM being decreased from 42% to 21% and that at 0.3 nM from 90% to 70%. At maximal concentrations of glucagon, insulin had no effect. These results agree favourably with those previously reported (Hue *et al.*, 1978; Blackmore *et al.*, 1979; Strickland *et al.*, 1980). A similar response was obtained when the cells were challenged with the α -adrenergic agonist, phenylephrine. The presence of insulin increased the concentration of effector required to produce a half-maximal activation of phosphorylase from 0.3 to 0.6 μM (results not shown). Addition of [Leu]enkephalin to the cells resulted in a dose-dependent activation of phosphorylase over the concentration range 0.1–10 μM , with a half-maximal stimulation at 0.96 μM (Fig. 1). Prior addition of insulin had no significant effect on the stimulation of phosphorylase activity at any concentration of the opioid peptide tested ($P > 0.05$). To ensure that the apparent inability of insulin to inhibit the effects of the enkephalin was not simply a reflection of the incubation conditions chosen, these experiments were repeated by measuring phosphorylase *a* activity at different times between 2 and 10 min after addition of the enkephalin, and also after different preincubation times with insulin. Irrespective of whether insulin was added simultaneously with the opioid peptide or at different time intervals up to 10 min before the [Leu]enkephalin, insulin was unable to antagonize the effect (results not shown). This suggests that the actions of the enkephalin on carbohydrate mobilization are similar to those of angiotensin, the effect of which over the concentration range 1 nM–0.1 μM was also not antagonized by insulin (results not shown).

It has previously been demonstrated that insulin is unable to antagonize the stimulation of phosphatidylinositol turnover in liver by either phenylephrine (Thomas & Williamson, 1983) or vasopressin (Kirk *et al.*, 1981). As both the effects of [Leu]enkephalin (Leach *et al.*, 1986) and angiotensin (Creba *et al.*, 1983; Thomas *et al.*, 1984; Lynch *et al.*, 1985; Charest *et al.*, 1985) on hepatic carbohydrate metabolism are believed to occur as a result of inositol lipid turnover, these findings are consistent with the proposed mechanism of action of the opioid peptide. These results also provide further evidence that the opiates are not acting through catecholamine receptors in the liver, as postulated for morphine by Wong *et al.* (1977, 1978), as insulin would be expected to inhibit the action of the enkephalin before the hydrolysis of phosphatidylinositol 4,5-bisphosphate, as previously described after stimulation by phenylephrine (Thomas & Williamson, 1983).

Effect of [Leu]enkephalin, glucagon and angiotensin on cyclic AMP formation in isolated hepatocytes

Previous studies using neuronal tissue have indicated that the opioid peptides, particularly the enkephalins, induce a receptor-mediated decrease in the intracellular concentrations of cyclic AMP (Minneman & Iversen, 1976; Klee *et al.*, 1976; Wolleman *et al.*, 1979). However, it has also been demonstrated that [Leu]enkephalin, the enkephalin analogue [D-Ala², MePhe⁴, Met(O)ol]-enkephalin and dynorphin-(1–13) enhance cyclic AMP formation in a number of peripheral tissues, including adipocyte plasma membranes (Jean-Baptiste & Rizack, 1980), isolated islets of Langerhans (Green *et al.*, 1983a,b) and isolated guinea-pig enterocytes (Lopez-Ruiz *et al.*, 1985). Therefore a general stimulatory role for the enkephalins and dynorphin on cyclic AMP formation in non-neuronal tissues has been suggested (Lopez-Ruiz *et al.*, 1985). In the liver, β -endorphin has been shown to increase carbohydrate mobilization directly as a result of an activation of adenylate cyclase and an increased formation of cyclic AMP (Matsumura *et al.*, 1984; Dave *et al.*, 1985). However, in contrast, our preliminary studies have suggested that the effects of the enkephalins on cyclic nucleotide metabolism in liver are more comparable with those in neuronal tissue (Leach & Titheradge, 1986). Experiments were therefore carried

out to characterize further this effect of [Leu]enkephalin. Table 2 shows the effect of $10\ \mu\text{M}$ -[Leu]enkephalin on cyclic AMP formation and phosphorylase *a* activity in isolated hepatocytes incubated in both the absence and the presence of $1\ \text{nM}$ -glucagon. The effect of the enkephalin was compared with that of the other Ca^{2+} -dependent agonist, angiotensin. It is evident that addition of maximal concentrations of all the effectors produced a comparable stimulation of phosphorylase *a* activity and that there was no significant degree of additivity with any combination. In agreement with our preliminary findings (Leach & Titheradge, 1986), addition of [Leu]enkephalin resulted in a small but significant decrease (18%) in the basal concentration of cyclic AMP in the cells plus medium ($P < 0.005$). It also resulted in a much larger (50%) decrease in the magnitude of the rise in cyclic AMP observed after addition of $1\ \text{nM}$ -glucagon to the cells ($P < 0.05$). Table 2 also compares this effect on cyclic AMP production with that of angiotensin, an agent known to decrease adenylate cyclase activity and the glucagon-induced rise in cyclic AMP in isolated hepatocytes (Jard *et al.*, 1981; Cardenas-Tanus *et al.*, 1982; Crane *et al.*, 1982; Keppens & De Wulf, 1984; Gunther, 1984; Pobiner *et al.*, 1985). It is evident that there was no significant difference, either qualitatively or quantitatively, between magnitude of the response to these Ca^{2+} -dependent agonists on the glucagon-stimulated increase in cyclic AMP production ($P > 0.05$). Similarly, there was no difference in the magnitude of their effects on basal cyclic AMP concentration, nor was the presence of both agents additive. The ability of angiotensin to inhibit basal cyclic AMP formation in hepatocytes is in contrast with that reported by others (Cardenas-Tanus *et al.*, 1982; Crane *et al.*, 1982; Morgan *et al.*, 1983); however, it is compatible with reports of an inhibition of basal adenylate cyclase activity in purified liver plasma membranes (Jard *et al.*, 1981; Crane *et al.*, 1982; Pobiner *et al.*, 1985).

Fig. 2 shows the effect of [Leu]enkephalin with time on the cyclic AMP concentrations in the cells incubated in the presence and absence of $1\ \text{nM}$ -glucagon. After addition of glucagon, there was a rapid rise in cyclic AMP formation, which reached a maximum within 2 min. This concentration was maintained over the next 2 min and gradually declined to 80% of the maximal response over the next 8 min. The presence of $10\ \mu\text{M}$ -[Leu]enkephalin decreased the increase in cyclic AMP induced by glucagon at all time points measured. The volume at 2 min was only 76% of the maximal concentration achieved in the absence of the enkephalin. There was no obvious plateau, and an immediate, more rapid, rate of decline towards basal values was evident, such that by 10 min the concentration of cyclic AMP was only 46% of that measured in the presence of glucagon alone.

Fig. 3 shows the effect of the enkephalin on the dose-dependency for the rise in cyclic AMP 2 min after addition of increasing concentrations of glucagon. At concentrations of glucagon up to $0.1\ \text{nM}$, there was a small rise in cyclic AMP formation, the concentration at $0.1\ \text{nM}$ being 140% of the control. Although this was lowered by addition of the opioid peptide, it was not statistically significant. As the concentration of glucagon increased between 0.1 and $1\ \text{nM}$, there was a dramatic increase in cyclic AMP formation, the concentration in the cells plus medium being increased 6.8-fold at $0.3\ \text{nM}$

Fig. 2. Time course of the effect of [Leu]enkephalin on the stimulation of cyclic AMP formation by $1\ \text{nM}$ -glucagon in isolated hepatocytes

After a 30 min incubation period, [Leu]enkephalin ($10\ \mu\text{M}$), glucagon ($1\ \text{nM}$) or both were added to the cell incubations as appropriate. Samples were removed at the times indicated for the determination of phosphorylase *a* activity as described in the Experimental section. The results shown are means of determinations on three separate cell preparations. Symbols: ○, control incubation; ●, [Leu]enkephalin; □, glucagon; ■, glucagon plus [Leu]enkephalin.

and 13-fold at $1\ \text{nM}$. Inclusion of $10\ \mu\text{M}$ -[Leu]enkephalin in the incubation consistently decreased the effect of the glucagon, and decreased the formation of cyclic AMP by 60% and 43% at $0.3\ \text{nM}$ - and $1\ \text{nM}$ -glucagon respectively. These effects of the opioid peptide are comparable with those previously published for the inhibition of adenylate cyclase activity by angiotensin (Crane *et al.*, 1982; Morgan *et al.*, 1983; Gunther, 1984) and emphasize the similarity in the mechanism of action of the two agents. In the series of experiments in Fig. 2 there was no significant effect of [Leu]enkephalin on the basal concentration of cyclic AMP in the cells, whereas the data in Table 2 and Fig. 3 show an 18% and 17% inhibition respectively, indicating that the ability of the enkephalin to decrease basal concentrations may depend on the original state of the animals from which the cells were prepared. This may explain the discrepancy between the ability of angiotensin to decrease basal concentrations of cyclic AMP, described in Table 1, and previous reports (Cardenas-Tanus *et al.*, 1982; Crane *et al.*, 1982; Morgan *et al.*, 1983).

Effect of [Leu]enkephalin and angiotensin on adenylate cyclase activity in liver plasma membranes

Although the above results indicate that [Leu]enkephalin is capable of decreasing the concentration

Fig. 3. Effect of [Leu]enkephalin on the stimulation of cyclic AMP formation by various concentrations of glucagon

After a 30 min preincubation period, [Leu]enkephalin (10 μ M) or vehicle was added to the cells together with glucagon at the concentrations indicated. Samples were removed for the determination of cyclic AMP after a further 2 min of incubation. The results are the mean values for three separate cell preparations. Symbols: $\square$, glucagon; $\blacksquare$, glucagon plus [Leu]enkephalin.

of cyclic AMP in hepatocytes, the results need not necessarily imply an inhibition of adenylate cyclase activity; the effect of the enkephalin could equally well be explained solely by an inhibition of phosphodiesterase activity. Indeed, from previous work with the Ca^{2+} -dependent agonists in liver, it is apparent that both of these effects may occur after treatment of hepatocytes with angiotensin, whereas vasopressin appears to act predominantly at the level of the phosphodiesterase, with no apparent inhibition of adenylate cyclase activity (Cantau *et al.*, 1980; Jard *et al.*, 1981; Keppens & De Wulf, 1984). Therefore we examined the effect of both [Leu]enkephalin and angiotensin on adenylate cyclase activity in purified liver plasma membranes in both the absence and the presence of increasing concentrations of glucagon, and in the presence of 3-isobutyl-1-methylxanthine to inhibit phosphodiesterase activity. The basal adenylate cyclase activity measured in the presence of 100 mM-NaCl and 0.1 mM-GTP was 225 ± 28 pmol/10 min per mg of protein ($n = 8$). Addition of 1 μ M-angiotensin to the incubation medium decreased the basal activity to 164 ± 18 pmol/10 min per mg of protein ($n = 6$). Both the basal activity and the degree of inhibition by angiotensin agreed favourably with values previously obtained by similar methods, indicating that the adenylate cyclase in our membrane preparation was hormone-sensitive and of purity comparable with those used previously (Jard *et al.*, 1981; Crane *et al.*, 1982; Pobiner *et al.*, 1985). Treatment of the membranes with 10 μ M-[Leu]enkephalin also significantly decreased ($P < 0.05$) the basal adenylate cyclase activity by 29%, to 160 ± 17 pmol/10 min per mg of protein ($n = 6$). This degree of inhibition was comparable in magnitude with the inhibition of basal adenylate cyclase activity in neuroblastoma $\times$ glioma NG108-15 cells by

Fig. 4. Comparison of the effects of [Leu]enkephalin and angiotensin on the inhibition of glucagon-stimulated adenylate cyclase activity of purified plasma membranes

Liver plasma membranes prepared in the presence of EDTA were incubated for 10 min at 30 $^{\circ}\text{C}$ in the presence of 100 mM-NaCl and 0.1 mM-GTP as described in the Experimental section. Glucagon was added to the final concentrations indicated, together with 1 μ M-angiotensin, 10 μ M-[Leu]enkephalin or vehicle. The results are expressed as means $\pm$ S.E.M. for eight different experiments with three separate membrane preparations. Symbols: $\square$, glucagon; $\bullet$, glucagon plus angiotensin; $\blacksquare$, glucagon plus [Leu]enkephalin.

[Leu]enkephalin (Klee *et al.*, 1976) and was not significantly different from that obtained with angiotensin ($P > 0.05$). Fig. 4 shows the effect of both angiotensin and [Leu]enkephalin on the increase in activity of adenylate cyclase in the appropriate control membranes incubated with different concentrations of glucagon. The results indicate that both [Leu]enkephalin and angiotensin decreased the ability of glucagon to stimulate adenylate cyclase activity over the entire concentration range of glucagon tested. There was no significant difference between the effects of either agent, both peptides being equally effective ($P > 0.05$). The percentage effects of both [Leu]enkephalin and angiotensin were greater at submaximal concentrations of glucagon, the inhibition being approx. 50% and 60% at 1 nM-glucagon respectively. However, the inhibition was still evident at maximal concentrations of glucagon, the effect of 1 μ M-glucagon on adenylate cyclase activity being lowered by approx. 19% with both of the Ca^{2+} -dependent agonists. This is in good agreement with previous determinations of the effect of angiotensin on glucagon-stimulated adenylate cyclase activity in liver plasma membranes (Jard *et al.*, 1981; Crane *et al.*, 1982). Therefore the results indicate that the effects of [Leu]enkephalin on hepatic cyclic nucleotide metabolism are comparable with those of angiotensin, but distinct from that of the other vasoactive peptide, vasopressin, in that the decrease in cyclic AMP formation is the result of an inhibition of

adenylate cyclase activity, in addition to any possible effects on phosphodiesterase activity.

From the above results, it is evident that addition of the opioid peptide [Leu]enkephalin to isolated hepatocytes induces a response comparable with that of the vasoactive peptide angiotensin. The effects of both peptides are insulin-insensitive and are the result of a stimulation of inositol lipid turnover and an increase in cytosolic Ca^{2+} (the present work; Leach *et al.*, 1986). In addition, both effectors lower the intracellular concentration of cyclic AMP in isolated hepatocytes and decrease adenylate cyclase activity in liver plasma membranes both under basal conditions and after stimulation with sub-optimal concentrations of glucagon. The only differences in response that are apparent are the magnitude of the stimulation of glucose formation from $[2\text{-}^{14}\text{C}]$ pyruvate plus lactate and the ability of [Leu]-enkephalin to decrease lactate formation by the hepatocytes (Leach *et al.*, 1985), where the effects of the opioid peptide more closely resemble those of glucagon.

From specificity studies, we have previously suggested that, if the opioid peptides are acting through specific opiate receptors in the liver, they must be similar to the δ -receptors identified in neuronal tissue (Allan *et al.*, 1983). The results presented above would confirm this supposition, as it is evident that the ability of the opiates to decrease cyclic AMP and to inhibit both basal and hormone-stimulated adenylate cyclase activity in neuroblastoma $\times$ glioma hybrid cell lines is also the result of the opiates binding to δ -type opiate receptors (reviewed by Blume, 1983), although other opiate receptors may also be negatively coupled to adenylate cyclase in different locations within the brain (Clark *et al.*, 1986). From the concentration-dependence of these effects of the opioid peptides, it is evident that the liver would not be responsive to the circulating concentrations of enkephalins in the blood (Frederickson & Geary, 1982); however, there is evidence to suggest that they may be synthesized and released locally. Wadja *et al.* (1976) first demonstrated that substances with opiate-like activity are present in the liver. We have subsequently confirmed that both perfused liver and isolated hepatocytes contain endogenous substances with the ability specifically to displace radiolabelled ligands from μ -, δ - and κ -receptors in brain membranes, and have positively identified by radioimmunoassay at least one of these compounds as [Met]enkephalin (Khawaja *et al.*, 1987). It would seem unlikely that these peptides have been internalized from the circulation and concentrated by the liver, as we have demonstrated that, as with other tissues responsive to opioid peptides, both hepatocytes and purified liver plasma membranes contain a very active aminopeptidase activity which destroys receptor binding and terminates the response by removing the *N*-terminal tyrosine residue from the enkephalins (Leach *et al.*, 1985).

This work was supported by grants from the British Diabetic Association and Wellcome Trust. R.P.L. thanks the Scientific and Engineering Research Council for a research studentship.

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Received 1 June 1987/24 August 1987; accepted 25 September 1987