The stimulation of glycogenolysis in isolated hepatocytes by opioid peptides

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Addition of the opioid peptides, [Leu]enkephalin and [Met]enkephalin, to isolated hepatocytes was shown to produce a stimulation of glycogenolysis comparable with that observed in the presence of maximal concentrations of glucagon, adrenaline or angiotensin. This stimulation was demonstrated to be the result of an activation of phosphorylase by a rapid Ca^{2+} -dependent mechanism and was not decreased by the presence of either α - or β -adrenergic antagonists, although it was dependent on the presence of the *N*-terminal tyrosine residue in the enkephalin molecule. It is suggested that this may be further evidence for specific opioid receptors in the liver. Addition of [Leu]enkephalin also inhibited lactate formation, indicating that the opioid peptides exert a concerted effect on hepatic carbohydrate metabolism to enhance glucose output. The transient nature of the effect of the enkephalins was shown to be the result of a rapid breakdown of the peptides in the incubation as a result of aminopeptidase activity, the initial product being the inactive des-tyrosine derivative.

Although it has been established for many years that opiates exert considerable effects on hepatic carbohydrate metabolism, this has mainly been ascribed to an indirect action of the opiates, as a result of either activation of the sympathetic nervous system (Feldberg & Gupta, 1974; Dey & Feldberg, 1975) or alterations in the circulating plasma concentrations of glucagon and insulin (Ipp *et al.*, 1978, 1980, 1982; Reid & Yen, 1981). However, in addition to these indirect effects, there is now evidence to suggest that opioid peptides may influence carbohydrate metabolism directly. We have demonstrated that addition of [Leu]enkephalin or dynorphin-(1–13) to isolated hepatocytes resulted in an acute, dose-dependent, activation of both glycogenolysis and formation of glucose from $[2\text{-}^{14}\text{C}]\text{pyruvate}$ (Allan *et al.*, 1983). This response was restricted to opioid peptides, morphine being ineffective, and was comparable in magnitude with that of either glucagon or the catecholamines. In addition to these peptides, Matsumura *et al.* (1984) have demonstrated that β -endorphin is capable of stimulating both glycogenolysis and gluconeogenesis from lactate in hepatocytes from fed and starved rats respectively and have suggested that this may in part explain the sustained hyperglycaemia noted after intravenous infusion of β -endorphin into the rat. Although these responses have not been well characterized, the effect of β -endorphin appears to act via a cyclic

AMP-dependent mechanism (Matsumura *et al.*, 1984), whereas the stimulation of glycogenolysis by [Leu]enkephalin was independent of alterations in cyclic AMP production (Allan *et al.*, 1983). This suggests that several opiate-receptor subtypes may be present in liver and may be involved in regulating hepatic glucose mobilization. The experiments in the present study were designed to characterize further the effect of enkephalins on glycogenolysis in hepatocytes.

Experimental

Materials

[Leu]enkephalin, angiotensin, adrenaline bitartrate, dihydroergotamine tartrate, DL-propranolol, collagenase and bacitracin were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Glucagon was a gift from Novo Laboratories (Basingstoke, Hants., U.K.). $[U\text{-}^{14}\text{C}]\text{Glucose 1-phosphate}$ was obtained from Amersham International (Amersham, Bucks., U.K.). All other chemicals either were of AnalaR grade from BDH Chemicals (Poole, Dorset, U.K.) or were from Sigma Chemical Co. (Poole, Dorset, U.K.).

Methods

Hepatocytes were prepared from fed male Sprague–Dawley rats weighing 180–240 g essen-

tially 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, containing 1.5% (w/v) gelatin, to a final concentration of approx. 60 mg wet wt./ml and incubated at 37°C under an atmosphere of O₂/CO₂ (19:1) in the presence of 0.1% (w/v) bacitracin. When the effects of cellular Ca²⁺ depletion were examined, the cells were washed in Ca²⁺-free Krebs–Henseleit bicarbonate buffer containing 1 mM-EGTA and gelatin, and the incubation was carried out in this buffer (Assimakopoulos-Jeannet *et al.*, 1977). The final incubation volume was 2 ml, unless stated otherwise, and the incubation period with the hormones or enkephalins was as stated in the text. All hormones and peptides were added as 100-fold concentrates in 0.9% NaCl containing 1 mg of bovine serum albumin/ml. Samples of the cells plus incubation media were removed just before the addition of the hormones and also at the end of the incubation period. These were deproteinized in trichloroacetic acid (final concn. 10%, w/v) and the rate of glycogenolysis was determined by the output of glucose into the medium by using glucose oxidase (Bergmeyer & Bernt, 1974). For the determination of lactate output by the cells, the samples were deproteinized with 2.9 M-HClO₄ and neutralized with KHCO₃ before assay (Gutmann & Wahlefeld, 1974). Phosphorylase *a* activity was measured in the media described by Hue *et al.* (1975), except that the formation of glycogen was monitored by the incorporation of [U-¹⁴C]glucose 1-phosphate into glycogen as described by Gilboe *et al.* (1972). For this, 0.2 ml of the cell suspension was added to 0.8 ml of buffer containing 100 mM-NaF, 20 mM-EDTA, 0.5% (w/v) glycogen and 50 mM-glycylglycine, pH 7.4. The samples were immediately frozen in liquid N₂, then thawed and sonicated for 5 s just before assay, with an M.S.E. ultrasonic disintegrator mark II at an amplitude of 12 μ m. The assay buffer contained 100 mM-[U-¹⁴C]glucose 1-phosphate (sp. radioactivity 0.0025 Ci/mol), 2% (w/v) glycogen, 0.3 mM-NaF and 1 mM-caffeine, pH 6.1. Where appropriate, α - or β -adrenergic antagonists were added 5 min before the addition of the catecholamine or enkephalin. Measurements of enkephalin degradation were carried out in cells incubated in Krebs–Henseleit bicarbonate buffer, pH 7.4. The enkephalins were added to the cell incubation to a final concentration of 100 μ M and, at the appropriate time, samples were removed and immediately centrifuged at 10000 *g* for 15 s in an Eppendorf micro-centrifuge. The supernatants were rapidly poured off and frozen in liquid N₂. The supernatants were then analysed by h.p.l.c. on a Spherisorb C18 column (HPLC Technology, Maccles-

field, Cheshire, U.K.) in 0.08% H₃PO₄, pH 2.5, with a linear gradient of 0–40% (v/v) acetonitrile.

Results are expressed as means $\pm$ S.E.M. Statistical analysis of the results was done by a paired Student's *t* test.

Results and discussion

Effect of [Leu]enkephalin on the rate of glycogenolysis and lactate formation in isolated hepatocytes

In a previous study, we have shown that treatment of hepatocytes with [Leu]enkephalin results in a dose-dependent stimulation of both gluconeogenesis and total glucose output by the cells (Allan *et al.*, 1983). Fig. 1(a) shows the time course of the effect of [Leu]enkephalin on glycogenolysis in comparison with those of glucagon and angiotensin. Over the first 10 min of the incubation, addition of the opioid peptide increased glucose output 2.6-fold. This stimulation was comparable with that produced by either glucagon or angiotensin, the rates of glucose output being 4.01 ± 0.50 , 10.40 ± 1.60 , 10.63 ± 1.26 and 9.98 ± 1.11 μ mol of glucose produced/min per g dry wt. of cells (*n* = 5) for controls, [Leu]enkephalin, glucagon and angiotensin treatment respectively. During the second 10 min incubation period, the magnitude of the effect of [Leu]enkephalin was gradually decreased until, at 20 min and beyond, the rate of glucose production was not significantly different from that in the control incubation (*P* > 0.05). This contrasts with the effects of glucagon and angiotensin, which remained significant over the first 30 min of incubation, and indicates that the effects of opioid peptides are more transient than those of the other hormones.

To gain more insight into the molecular mechanisms by which the opioid peptides influence hepatic carbohydrate metabolism, the concentration of lactate in the cells plus medium was also determined under the above glycogenolytic conditions. The time course is shown in Fig. 1(b). In agreement with previous studies (Hems *et al.*, 1978; Hue *et al.*, 1981a,b), the control cells showed a small steady increase in lactate production, whereas addition of glucagon produced a marked utilization of lactate by the cells, which was maintained over the entire incubation period. Addition of [Leu]enkephalin also significantly decreased lactate production over the first 10 min incubation period when compared with addition of vehicle alone (*P* < 0.002), the uptake of lactate being comparable with that produced by glucagon (*P* > 0.05). However, after this time, a net lactate output occurred which closely paralleled that of the control cells. Although angiotensin produced a stimulation of glycogenolysis over the entire incubation period comparable with that by either

Fig. 1. Effect of [Leu]enkephalin, glucagon and angiotensin on the rate of (a) glucose output and (b) lactate output by the cells. The experimental protocol was as described in the Experimental section. The effectors were added at zero time to final concentrations of $10\text{ }\mu\text{M}$, $1\text{ }\mu\text{M}$ and 1 nM for [Leu]enkephalin, angiotensin and glucagon respectively. Samples were removed for the determination of glucose release and the concentration of lactate in the cells plus medium at the times indicated. Results are means $\pm$ S.E.M. for six different cell preparations. Symbols: ○, control; ■, glucagon; ▲, angiotensin; ●, [Leu]enkephalin.

glucagon or [Leu]enkephalin, only a small, insignificant, utilization of lactate was observed over the first 20 min, confirming that the Ca^{2+} -dependent hormones do not significantly decrease glycolysis (Hems *et al.*, 1978; Hue *et al.*, 1981a,b). These results indicate that [Leu]enkephalin acts in an analogous manner to glucagon and suggest that the opioid peptides have a concerted effect on hepatic carbohydrate metabolism in the fed rat, stimulating both gluconeogenesis and glycogenolysis while simultaneously inhibiting glycolysis.

Specificity of the response to enkephalins

We have previously shown that glucose production by the liver can be enhanced by the opioid peptides [Leu]enkephalin and dynorphin-(1-13), but not by morphine (Allan *et al.*, 1983). Dynorphin-(1-13) contains the [Leu]enkephalin sequence as its *N*-terminal (Goldstein *et al.*, 1981), and therefore it was of interest to determine the extent of this sequence required for biological activity. The effect of [Met]enkephalin on glycogenolysis was also examined, as it is apparent that it is synthesized and released together with [Leu]enkephalin in a number of tissues, including sympathetic neurons and the adrenal medulla (Hughes *et al.*, 1977; Rossier *et al.*, 1980; Gubler *et al.*, 1982), and is also the *N*-terminal sequence of β -endorphin, a peptide also known to stimulate both gluconeogenesis and glycogenolysis (Matsumura *et al.*, 1984). The results are shown in Table 1. Replacement of leucine with methionine at residue 5 to form [Met]enkephalin retained biological activity within the molecule, suggesting that both

Table 1. Specificity of the glycogenolytic response to enkephalins

The cells were preincubated for 30 min as described in the Experimental section before the addition of the peptides (final concn. $10\text{ }\mu\text{M}$). Glucose output was measured over the next 15 min. Results are expressed as means $\pm$ S.E.M. with the numbers of cell preparations given in parentheses. * $P < 0.001$ for significance of difference from control.

Additions	Glucose release ($\mu\text{mol/min per g dry wt.}$)
Vehicle	2.59 ± 0.50 (8)
[Met]enkephalin	5.25 ± 0.66 (8)*
Des-Tyr-[Leu]enkephalin	3.31 ± 0.42 (5)
Glycylglycylphenylalanine	3.24 ± 0.73 (8)
Glycylphenylalanine	3.40 ± 0.47 (7)

natural enkephalins may be involved in the acute regulation of hepatic carbohydrate metabolism. The *N*-terminal tyrosine residue of [Leu]enkephalin was essential for the maintenance of glycogenolytic activity; addition of des-Tyr-[Leu]enkephalin to the cells produced no significant change in glycogen breakdown when compared with effects of vehicle alone or smaller fragments of the enkephalin molecule ($P > 0.05$). This is further evidence in favour of the concept that the opioid peptides are in fact acting at opiate receptors within the liver, as it has been established in other tissues that the presence of tyrosine residue in the molecule is essential for opiate-receptor binding (Kosterlitz *et al.*, 1980).

Effect of [Met]enkephalin on phosphorylase *a* activity

Previous studies have indicated that the stimulation of glycogenolysis by both glucagon and the Ca^{2+} -dependent hormones is the result of protein phosphorylation and an increase in phosphorylase *a* activity within the cells (Hems *et al.*, 1976; Keppens & De Wulf, 1976; Keppens *et al.*, 1977; Hutson *et al.*, 1976; Cherrington *et al.*, 1976). To determine whether the enkephalins act via a similar mechanism or whether the enhanced glucose output is either the result of an allosteric activation of phosphorylase *b* or non-phosphorylytic glycogenolysis, the effect of [Met]enkephalin on phosphorylase *a* was determined at various time intervals after addition of the peptide to the cells. The results (Fig. 2) show that the increased glycogen breakdown induced by the enkephalins was also associated with an increase in phosphorylase *a* activity. The time of onset of the response was extremely rapid, an activation being apparent within 15 s after addition of the peptide to the cells. The maximal activity was achieved within 1 min and the response was sustained over 5 min. In agreement with the magnitude of the effect of the

Fig. 2. Time course of the activation of phosphorylase by [Met]enkephalin

The hepatocytes were preincubated for 30 min before the addition of vehicle or [Met]enkephalin (final concn. $10 \mu\text{M}$). The final incubation volume was 4 ml. Samples were removed at the times indicated and immediately processed as described in the Experimental section. Results are means $\pm$ S.E.M. for six different cell preparations. Symbols: $\circ$, control; $\bullet$, [Met]enkephalin.

enkephalins on glycogenolysis (Fig. 1a), the maximal activity obtained in the presence of [Met]enkephalin was comparable with that obtained with both glucagon and angiotensin. The activities measured 2 min after the addition of the peptides to the cells were 108.6 ± 12.2 , 181.1 ± 14.5 , 182.9 ± 11.7 and 206.3 ± 17.8 units/g dry wt. for control cells and cells treated with [Met]enkephalin, glucagon and angiotensin respectively ($n = 6$, $P < 0.001$ for difference between activities in control and hormone-treated cells).

Effect of α - and β -adrenergic antagonists on the glycogenolytic effect of [Leu]enkephalin

Wong *et al.* (1974, 1978) have suggested that the long-term effects of opiates on carbohydrate and lipid metabolism at the level of the liver may be through a direct interaction of the opiate with catecholamine receptors. The short-term effects of opioid peptides also resemble those of the catecholamines in that they are mediated by a cyclic AMP-independent mechanism and show a similar time of onset and dependence on the concentration of effector (Garrison & Haynes, 1973; Keppens *et al.*, 1977; Assimacopoulos-Jeannet *et al.*, 1977; Allan *et al.*, 1983). Therefore we have compared the ability of α - and β -adrenergic antagonists to inhibit the stimulation of glycogenolysis by [Leu]enkephalin and adrenaline. The results are shown in Table 2. Both [Leu]enkephalin and adrenaline produced a comparable stimulation of glucose output when added to the cells. Prior incubation of the cells with either the β -adrenergic antagonist, DL-propranolol, or the α -adrenergic antagonist, dihydroergotamine, had no significant effect on either the basal rate of glycogen degradation or the stimulated rate in the presence of [Leu]enkephalin. In contrast, dihydroergotamine abolished the effect of adrenaline, which is consistent with the response being via α -adrenergic receptors (Tolbert *et al.*, 1973; Hutson *et al.*, 1976; Assimacopoulos-Jeannet *et al.*, 1977). Thus the results indicate that [Leu]enkephalin does not act by an α -adrenergic receptor. Binding of the opioid peptide to the β -receptor would also appear unlikely, as DL-propranolol was without effect and the response was independent of an increase in cyclic AMP (Allan *et al.*, 1983).

Effect of cellular Ca^{2+} depletion on the stimulation of glycogenolysis by [Leu]enkephalin

The possible role of Ca^{2+} in the stimulation of glycogenolysis by enkephalins was investigated by depleting the cells of Ca^{2+} by incubation in Ca^{2+} -free buffer containing 1 mM-EGTA, as described by Assimacopoulos-Jeannet *et al.* (1977). Table 3 shows a comparison of the effect of [Leu]enkephalin with those of glucagon, angiotensin and adrenaline in normal and Ca^{2+} -free buffer. As

Table 2. *Effect of α - and β -adrenergic antagonists on the stimulation of glycogenolysis by [Leu]enkephalin and adrenaline*
The experimental protocol was as described in the Experimental section. The cells were preincubated for 10 min before the addition of the α - and β -adrenergic antagonists (final concns. 20 μ M). After a further 5 min incubation period, the [Leu]enkephalin and adrenaline were added (final concns. 10 μ M and 1 μ M respectively), and the output of glucose was measured over the next 15 min. Results are expressed as means $\pm$ S.E.M. for five different cell preparations. * $P < 0.001$ for significance of difference from appropriate controls.

Additions	Glucose output (μ mol/min per g dry wt.)		
	Vehicle	Propranolol	Dihydroergotamine
Control	4.82 $\pm$ 0.46	4.43 $\pm$ 0.43	4.55 $\pm$ 0.33
Adrenaline	12.86 $\pm$ 1.39*	12.58 $\pm$ 1.57*	6.61 $\pm$ 0.68
[Leu]enkephalin	12.11 $\pm$ 0.80*	11.52 $\pm$ 0.50*	11.67 $\pm$ 0.56*

Table 3. *Effect of Ca^{2+} depletion on glycogenolysis in hepatocytes incubated in the presence of [Leu]enkephalin, glucagon, angiotensin and adrenaline*

Hepatocytes were incubated for 15 min either in normal Krebs–Henseleit bicarbonate buffer or in Ca^{2+} -free buffer containing 1 mM-EGTA as described in the Experimental section. The effectors were added to the final concentrations indicated and glucose release was measured over the next 20 min. Results are expressed as means $\pm$ S.E.M. for 11 different cell preparations. * $P < 0.001$ for difference from appropriate controls.

Additions	Glucose output (μ mol/min per g dry wt.)	
	2.5 mM- Ca^{2+}	1 mM-EGTA
Control	2.85 $\pm$ 0.25	2.02 $\pm$ 0.22
[Leu]enkephalin (10 μ M)	6.27 $\pm$ 0.76*	2.72 $\pm$ 0.37
Glucagon (1 nM)	8.51 $\pm$ 0.90*	6.89 $\pm$ 0.95*
Angiotensin (1 μ M)	5.86 $\pm$ 0.65*	2.60 $\pm$ 0.45
Adrenaline (1 μ M)	6.87 $\pm$ 0.82*	6.04 $\pm$ 0.80*

shown in Fig. 1(a) and Table 2, all four agonists significantly increased the rate of glycogenolysis in control cells ($P < 0.001$). Depleting the cells of Ca^{2+} lowered the rate of glycogenolysis in the absence of hormones by 29% ($P < 0.05$) and decreased the stimulation of glucose output by [Leu]enkephalin to a value which overall was not significantly different from that of the control cells. The stimulation by [Leu]enkephalin in the presence and absence of Ca^{2+} was 219 ± 19 and $142 \pm 20\%$ respectively. The effects of Ca^{2+} depletion on the stimulation of glycogenolysis by glucagon, adrenaline and angiotensin were as described previously by other workers (Stubbs *et al.*, 1976; Assimacopoulos-Jeannet *et al.*, 1977; Keppens *et al.*, 1977; Van de Werve *et al.*, 1977; Chan & Exton, 1977), with only angiotensin showing a significant diminution of the response. This was comparable with that observed with [Leu]enkephalin, the percentage stimulation of glucose output by angiotensin

being decreased from 207 ± 19 to 133 ± 24 by omission of Ca^{2+} . This suggests that [Leu]enkephalin acts via a similar mechanism to that of angiotensin and vasopressin, presumably via a mobilization of intracellular Ca^{2+} , and provides further evidence that the enkephalins are not acting via the catecholamine or glucagon receptors (Wong *et al.*, 1974, 1978).

Degradation of [Leu]enkephalin by isolated hepatocytes

From the time course of action of [Leu]enkephalin on both glycogenolysis and lactate uptake, it is apparent that the effect of the opioid peptides are of short duration (Figs. 1a and 1b). The enkephalins are known to be rapidly degraded by number of amino- and endo-peptidases in other tissues (for review see Schwartz, 1983), and enkephalin degradation has been reported to occur in liver homogenates (Shaw *et al.*, 1979). Therefore the stability of the enkephalin in the incubation medium was measured by h.p.l.c. to determine whether peptide hydrolysis may explain the transient nature of the response. Initial experiments using both 10 μ M-[Leu]enkephalin and -[Met]enkephalin demonstrated that in the absence of hepatocytes there was no loss of either peptide from the medium over 5 min of incubation, whereas when cells were present none of the peptides could be detected in the incubation medium after 1 min (results not shown). To facilitate more accurate measurements of enkephalin degradation, the concentration of [Leu]enkephalin was increased to 100 μ M in the incubation. The results are shown in Fig. 3. Even at this high concentration of enkephalin there was a rapid loss of the peptide from the incubation medium, and this was paralleled by the formation of the inactive derivative, des-Tyr-[Leu]enkephalin. Within 15 s of addition of the peptide to the cells, only 43% of the initial enkephalin was still detectable, and this was decreased to less than 5% within 2 min. The initial product, des-Tyr-[Leu]en-

Fig. 3. H.p.l.c. analysis of the concentration of [Leu]-enkephalin in the cell incubation medium

[Leu]enkephalin was added to the cell incubation medium to a final concentration of $100\mu\text{M}$. At the appropriate time, samples were removed, rapidly centrifuged and the supernatants frozen in liquid N_2 before assay for the peptides by h.p.l.c. as described in the Experimental section. A zero-time sample in which the [Leu]enkephalin was added to Krebs-Henseleit bicarbonate buffer in the absence of cells is shown in (a) and a des-Tyr-[Leu]enkephalin standard in (b). (c) and (d) show cell extracts at 15 s and 2 min after the addition of the peptide to the cells.

kephalin, was further degraded, and a number of unidentified peaks of smaller fragments appeared, which were not apparent in incubations in the absence of [Leu]enkephalin (results not shown).

These results confirm that liver has enzymes capable of degrading enkephalins and suggest that the major degradative enzyme is an *N*-terminal aminopeptidase, similar to the aminoenkephalinases described in brain and guinea-pig ileum (Geary *et al.*, 1982; Hui *et al.*, 1983). Preliminary experiments indicate that angiotensin is not degraded by this aminopeptidase activity, suggesting that it may be specific to enkephalins and thus a means for terminating the response *in vivo*. In addition to providing an explanation for the short duration of the effect of opioid peptides, the rapid degradation may also explain, in part at least, the high concentration of enkephalins required to elicit the response over longer-term incubations (Allan *et al.*, 1983), as it is apparent from studies with glucagon that the sensitivity of the response can be increased by several orders of magnitude by inhibition of proteolysis (Garrison & Haynes, 1973).

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