The stimulation of glycogenolysis in isolated hepatocytes by opioid peptides

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The opioid peptides [Leu]enkephalin and dynorphin-(1–13) were shown to enhance glycogen breakdown when added directly to hepatocytes. This was the result of a concerted effect on the enzymes of glycogen metabolism, with a stimulation of glycogen phosphorylase activity and a simultaneous decrease in glycogen synthase I activity. The latter only became significant when the enzyme was activated by incubating the cells in presence of 20 mM- or 40 mM-glucose. The effect of the opioid peptides was independent of an increase in cyclic AMP or any change in the activity ratio of the cyclic AMP-dependent protein kinase and was abolished by depleting the cells of Ca^{2+} . Both [Leu]enkephalin and dynorphin-(1–13) produced a significant decrease in cyclic AMP formation, suggesting that in liver, as in neuronal tissue, they may act by inhibiting adenylate cyclase activity.

INTRODUCTION

It has been known for many years that opiates cause profound hyperglycaemia when administered *in vivo* (Borison *et al.*, 1962; Feldberg & Gupta, 1974; Dey & Feldberg, 1975), but it has only recently been demonstrated that opioid peptides may influence carbohydrate metabolism directly at the level of the liver (Allan *et al.*, 1983; Matsumura *et al.*, 1984; Leach *et al.*, 1985). We have shown that addition of the opioid peptides [Leu]enkephalin, [Met]enkephalin and dynorphin-(1–13) to isolated hepatocytes has a rapid, dose-dependent, effect on carbohydrate metabolism, increasing the rate of glycogenolysis and glucose formation from [2- ^{14}C]pyruvate, while simultaneously inhibiting glycolytic flux (Allan *et al.*, 1983; Leach *et al.*, 1985). The effect of [Leu]enkephalin on glucose mobilization is comparable in magnitude with that produced by either the catecholamines or glucagon, and our initial work has suggested that this is via a Ca^{2+} -dependent mechanism. The response requires the presence of the *N*-terminal tyrosine residue of the molecule and cleavage of the peptide to form the inactive des-Tyr derivative by aminopeptidase activity provides a mechanism for rapidly terminating the response *in vivo* (Leach *et al.*, 1985). Similar studies by Matsumura and co-workers have demonstrated that β -endorphin also stimulates glycogenolysis and gluconeogenesis from lactate when added *in vitro* to hepatocytes, and induces hyperglycaemia *in vivo* (Matsumura *et al.*, 1984). However, in contrast with the effect of [Leu]enkephalin, β -endorphin was shown to act via a cyclic AMP-dependent mechanism, suggesting that there may be more than one receptor present in liver capable of interacting with opioid peptides. Evidence in favour of this has come from the demonstration of two specific binding sites for β -endorphin in hepatic membranes, at least one of which is functionally linked to adenylate cyclase (Dave *et al.*, 1985). The experiments in the present study were designed to characterize further the mechanism of action of the opioid peptides on the enzymes involved in glycogen metabolism.

EXPERIMENTAL

Materials

[Leu]enkephalin, dynorphin-(1–13), vasopressin, angiotensin II, 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- ^{14}C]Glucose 1-phosphate, UDP-[U- ^{14}C]glucose and [γ - ^{32}P]ATP were 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.

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) in the presence of 0.1% (w/v) bacitracin. When the effects of cellular Ca^{2+} depletion were examined, the cells were washed in Ca^{2+} -free Krebs–Henseleit bicarbonate buffer containing 1 mM-EGTA and the incubation was carried out in this buffer. The final incubation volume was 3 ml unless otherwise stated, and the incubation period with peptides or hormones was as described 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 were removed at the times stated in the text. The rate of glycogenolysis was determined by the loss of glycogen from the cells, by the method of Wood & Blum (1982). A 1 ml portion of cells was precipitated with 6 ml of ice-cold 85% (v/v) ethanol, and the cell pellet was obtained by centrifugation at 2000 *g* for 10 min. The pellet was washed twice with 95% ethanol and stored at –18 °C. After thawing, the glycogen content of the pellet was determined by the addition of 2 ml of 200 mM-sodium acetate buffer, pH 4.5, containing 2 mg of amyloglucosi-

dase/ml, and incubating the samples for 90 min at 45 °C. The glucose was then assayed by using glucose oxidase (Bergmeyer & Bernt, 1974). Phosphorylase activity was determined as described previously (Leach *et al.*, 1985). For the determination of cyclic AMP in the cells, the samples were deproteinized with 2.9 M-HClO₄, neutralized with KHCO₃ and stored at -20 °C before the cyclic AMP was determined by radioimmunoassay as described previously (Chisholm *et al.*, 1983). Cyclic AMP-dependent protein kinase activity was determined by the method of Cherrington *et al.* (1976) by measuring the incorporation of ³²P from [γ -³²P]ATP into histone protein and is expressed as the ratio of the activity measured in the presence and absence of 5 μ M-cyclic AMP. Glycogen synthase was assayed by the method of Thomas *et al.* (1968) by measuring the incorporation of UDP-[U-¹⁴C]-glucose into glycogen. The cells were incubated in the presence of 4 mM-pyruvate/16 mM-lactate plus a final concentration of 0, 20 mM- or 40 mM-glucose. At the time indicated, 0.4 ml of the cell suspension was removed and added to 0.4 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 frozen immediately in liquid N₂, then thawed and sonicated just before assay. During the present study it was evident that the cells exhibited different sensitivities to different batches of opioid peptides, with some batches being essentially inactive in our liver cell preparation. To determine whether this variability was a peculiarity of the hepatocyte system, the different batches were also tested for their ability to alter the secretion of insulin in rat islet incubations in the system of Green *et al.* (1983). In agreement with the study by Green *et al.* (1983), [Leu]enkephalin evoked a biphasic change in insulin secretion, with a stimulation at low concentrations and no effect at high concentrations, the rate of insulin secretion for a representative experiment being 1.78 ± 0.37 , 2.05 ± 0.58 , 3.29 ± 0.69 and 1.53 ± 0.56 ng of insulin released/30 min per islet for control, 1 ng, 1 μ g and 10 μ g of [Leu]enkephalin/ml respectively ($n = 8$). Those batches of enkephalin which failed to produce an effect in liver showed a clear shift in the dose-response to higher concentrations, and it was necessary to increase the concentration of the opioid peptide in the incubation 100-fold to produce a comparable response (results not shown). Analysis of the peptides by h.p.l.c. and u.v. spectra revealed no significant difference between batches and failed to show any contaminants which might account for the differences in efficacy. From physical studies, it is evident that the enkephalins may take up different configurations in solution and that the different conformers might bind to different receptor subtypes; thus it is conceivable that the peptides obtained from different sources may display this phenomenon (Renugopalakrishnan *et al.*, 1985).

Results are expressed as means $\pm$ S.E.M., with the numbers of different cell preparations given in parentheses. Statistical analysis of the results was done by a paired Student's *t* test.

RESULTS AND DISCUSSION

Effect of [Leu]enkephalin on phosphorylase α and glycogen breakdown

We have demonstrated previously that the opioid peptides [Leu]- and [Met]-enkephalin increase glucose

Fig. 1. Effect of [Leu]enkephalin on the rate of glycogen breakdown and phosphorylase activation

Hepatocytes were preincubated for 5 min before addition of vehicle or [Leu]enkephalin (final concn. 10 μ M). The final incubation volume was 15 ml. Samples were removed at the times indicated and immediately processed as described in the Experimental section. At 20 min a second portion of [Leu]enkephalin was added and the incubation continued for a further 10 min. Results expressed as means $\pm$ S.E.M. for seven different cell preparations. Symbols: $\circ$, control; $\bullet$, [Leu]enkephalin.

output when added directly to isolated hepatocytes (Allan *et al.*, 1983; Leach *et al.*, 1985). To demonstrate that the increased rate of glucose output correlated directly with an activation of phosphorylase by [Leu]enkephalin and with a consequent glycogen loss from the cells, hepatocytes from fed rats were treated with the peptide and samples were removed for the determination of both cell glycogen content and phosphorylase α activity. The results are shown in Fig. 1. It is evident that the stimulation of glucose output by [Leu]enkephalin described in previous studies correlates well with the loss of glycogen from the tissue (Fig. 1a), as a result of an increase in activity of phosphorylase α (Fig. 1b) on addition of 10 μ M-[Leu]enkephalin. Phosphorylase was maximally activated over the first 5 min after addition of the peptide, but gradually declined towards the basal activity over the next 15 min. The

change in phosphorylase *a* activity was reflected in the mobilization of glycogen from the cells, with a maximal loss occurring over the first 10 min, after which the rate was not significantly different from the controls. It has been demonstrated previously that other agents known to stimulate glycogen metabolism by a cyclic AMP-independent pathway become refractory and are incapable of responding to a second addition of effector under static incubation conditions (Breant *et al.*, 1981). To determine whether the response to [Leu]enkephalin was terminated as a result of the rapid breakdown of the enkephalin by aminopeptidase activity, as described previously (Leach *et al.*, 1985), or a desensitization of the cells to the opioid peptides, the cells were challenged with a second addition of a maximal concentration of [Leu]enkephalin at 20 min. No further increase in either glycogen breakdown or phosphorylase *a* activity was observed, reinforcing the similarity noted previously (Leach *et al.*, 1985) between the effects of [Leu]enkephalin on hepatocyte metabolism and those of vasopressin and α -adrenergic agents (Breant *et al.*, 1981).

Effect of [Leu]enkephalin and dynorphin on glycogen synthase I activity

Fig. 1 shows that the opioid peptides enhance glycogenolysis by increasing the percentage of phosphorylase in the form of phosphorylase *a* in the cell. However, to determine whether they have a concerted effect on the enzymes of glycogen metabolism, glycogen synthase I activity was also measured in hepatocytes exposed to [Leu]enkephalin and dynorphin-(1-13). The results are shown in Table 1. As described previously (Hue *et al.*, 1975), cells prepared from fed rats and incubated in the absence of glucose show an extremely low glycogen synthase I activity, with less than 9% of the total synthase activity measured in the presence of 10 mM-glucose 6-phosphate being expressed. After treatment of the cells with both the opioid peptides and glucagon, there was a small but insignificant decrease in glycogen synthase I activity. To increase the magnitude of the effect of hormone treatment on glycogen synthase I activity, the cells were incubated in the presence of increasing concentrations of glucose to activate the enzyme (Hue *et al.*, 1975; Hutson *et al.*, 1976) and samples were taken 5 min after the addition of the effectors. As the concentration of glucose in the

incubation was increased, glycogen synthase I activity in the control cells progressively increased until, at 40 mM-glucose, the percentage of the total glycogen synthase in the I form was 69%. At both 20 mM- and 40 mM-glucose, treatment with glucagon and the opioid peptides significantly decreased the glycogen synthase I activity, the rates in the presence of 40 mM-glucose being lowered to 34.0, 71.3 and 23.3% of the control activities after treatment with [Leu]enkephalin, dynorphin-(1-13) and glucagon respectively. The increase in activity of glycogen synthase in control cells by glucose addition was paralleled by a decrease in phosphorylase *a*, the activity being 112.5 ± 9.5 and 57.7 ± 15.5 ($n = 4$) at 0 and 20 mM-glucose respectively. No significant difference was observed in the stimulated rates, however, the phosphorylase *a* activities being 157.9 ± 5.4 and 154.8 ± 4.6 in the absence of glucose and 140.5 ± 7.7 and 141.1 ± 6.3 ($n = 4$) in the presence of glucose for treatment with [Leu]enkephalin and glucagon respectively. From these results, it is evident that the opioid peptides are capable of modulating the activity of both glycogen synthase and phosphorylase co-operatively such that glycogen degradation is favoured with respect to glycogen synthesis.

Effect of cellular depletion of Ca^{2+} on the opioid-peptide-mediated activation of phosphorylase

Previous studies have suggested that the effect of [Leu]enkephalin on glucose output is via a Ca^{2+} -dependent mechanism, as it can be abolished by depleting the cells of Ca^{2+} (Leach *et al.*, 1985). However, in view of the finding that β -endorphin stimulates both glycogenolysis and gluconeogenesis by binding to receptors coupled to the activation of adenylate cyclase (Matsumura *et al.*, 1984; Dave *et al.*, 1985) and that high concentrations of opioid peptides with *N*-terminal homologies to β -endorphin can compete with this binding (Dave *et al.*, 1985), we have re-investigated the potential role played by Ca^{2+} and cyclic AMP in the activation of phosphorylase by [Leu]enkephalin and compared the results with those obtained after treatment of the cells with [Met]enkephalin and dynorphin-(1-13), opioid peptides which are known to be capable of interacting with different opiate receptors from those favoured by [Leu]enkephalin, in other tissues (reviewed by Chang, 1984). Table 2 shows the effect of depleting the cells of Ca^{2+} by incubation in Ca^{2+} -free buffer containing

Table 1. Effect of [Leu]enkephalin, dynorphin-(1-13) and glucagon on glycogen synthase I activity in the presence of 0, 20 mM- and 40 mM-glucose

Hepatocytes were preincubated for 15 min in the presence of 0, 20 mM- or 40 mM-glucose (final concn.), before addition of effectors to final concentrations of 10 μM , 10 μM and 1 nM for [Leu]enkephalin, dynorphin-(1-13) and glucagon respectively. After a further 5 min, samples were removed from the incubation for the determination of glycogen synthase activity in the cells plus medium as described in the Experimental section. The total glycogen synthase activity in the cells plus medium in the presence of 10 mM-glucose 6-phosphate was 4.32 ± 0.84 $\mu\text{mol/min per g dry wt}$. Results are expressed as means $\pm$ S.E.M. for five different cell preparations: * $P < 0.05$ and ** $P < 0.005$ for difference from appropriate controls.

Addition	Glycogen synthase I activity ($\mu\text{mol/min per g dry wt.}$)		
	0 mM-glucose	20 mM-glucose	40 mM-glucose
Control	0.36 ± 0.05	1.08 ± 0.13	2.48 ± 0.27
[Leu]enkephalin (10 μM)	0.31 ± 0.01	$0.57 \pm 0.06^*$	0.85 ± 0.08
Dynorphin-(1-13) (10 μM)	0.33 ± 0.07	$0.75 \pm 0.14^*$	$1.77 \pm 0.27^*$
Glucagon (1 nM)	0.30 ± 0.03	$0.45 \pm 0.04^{**}$	$0.58 \pm 0.08^{**}$

Table 2. Effect of Ca²⁺ depletion on the stimulation of phosphorylase *a* activity by opioid peptides

Hepatocytes were preincubated for 30 min in either normal Krebs–Henseleit bicarbonate buffer or Ca²⁺-free buffer containing 1 mM-EGTA as described in the Experimental section. The effectors were added to the final concentrations indicated and phosphorylase *a* activity was determined at 5 min. Results are expressed as means $\pm$ S.E.M. for five different cell preparations: **P* < 0.05 and ***P* < 0.005 for difference from appropriate controls.

Addition	Phosphorylase <i>a</i> activity (μ mol/min per g dry wt.)	
	2.5 mM-Ca ²⁺	1 mM-EGTA (Ca ²⁺ -free)
Control	71.52 $\pm$ 3.23	52.48 $\pm$ 2.76
[Leu]enkephalin (10 μ M)	121.63 $\pm$ 4.05**	52.81 $\pm$ 2.49
[Met]enkephalin (10 μ M)	109.40 $\pm$ 15.20*	52.07 $\pm$ 3.49
Dynorphin-(1–13) (10 μ M)	112.20 $\pm$ 9.40*	55.90 $\pm$ 6.05
Vasopressin (100 nM)	116.10 $\pm$ 4.32**	51.63 $\pm$ 3.32
Glucagon (1 nM)	127.19 $\pm$ 3.00**	118.60 $\pm$ 4.70**

Table 3. Measurement of cyclic AMP concentrations, cyclic AMP-dependent protein kinase and phosphorylase *a* activity

Hepatocytes were preincubated for 30 min before addition of effectors to final concentrations of 10 μ M, 10 μ M and 1 nM for [Leu]enkephalin, dynorphin-(1–13) and glucagon respectively. Samples were removed from the incubation after 2 min for the determination of cyclic AMP concentrations, cyclic AMP-dependent protein kinase activity ratio and phosphorylase activity as described in the Experimental section. The total protein kinase activity measured in the presence of 5 μ M-cyclic AMP was 0.56 $\pm$ 0.07 μ mol of ³²P incorporated/min per g dry wt. and was not altered by the presence of the effectors. Results are expressed as means $\pm$ S.E.M. for six different cell preparations: **P* < 0.05 and ***P* < 0.005 for difference from appropriate controls.

Additions	Cyclic AMP (pmol/g dry. wt.)	Cyclic AMP-dependent protein kinase activity ratio (+ / – cyclic AMP)	Phosphorylase <i>a</i> activity (μ mol/min per g dry wt.)
Control	135.8 $\pm$ 14.1	0.572 $\pm$ 0.037	57.18 $\pm$ 3.61
[Leu]enkephalin	74.6 $\pm$ 7.5*	0.579 $\pm$ 0.021	93.80 $\pm$ 4.30**
Dynorphin-(1–13)	83.0 $\pm$ 8.2*	0.566 $\pm$ 0.018	79.30 $\pm$ 5.90*
Glucagon	1797.0 $\pm$ 180.0**	0.824 $\pm$ 0.025**	92.00 $\pm$ 7.40**

1 mM-EGTA (Assimakopoulos-Jeannet *et al.*, 1977) on the activation of phosphorylase after treatment of the cells with [Leu]enkephalin, [Met]enkephalin, dynorphin-(1–13), vasopressin and glucagon. In the presence of Ca²⁺, all the agonists tested produced a significant stimulation of phosphorylase activity, and there was no significant difference in the magnitude of stimulation produced by the addition of any of the effectors (*P* > 0.05). Depleting the cells of Ca²⁺ lowered the amount of phosphorylase *a* in the control cells by 26% and completely prevented the activation of phosphorylase by the opioid peptides. In agreement with previous studies, Ca²⁺-depletion also prevented the vasopressin-induced stimulation of phosphorylase, but did not affect that produced by glucagon (Stubbs *et al.*, 1976; Keppens *et al.*, 1976). These data suggest that, in contrast with β -endorphin, all of the opioid peptides studied act via a similar Ca²⁺-dependent mechanism, comparable with that of vasopressin, and bind to different receptors in liver from those of β -endorphin.

Effect of [Leu]enkephalin and dynorphin on cyclic AMP formation and the activity of the cyclic AMP-dependent protein kinase

To confirm that the enkephalins and dynorphin were acting independently of an increase in cytosolic cyclic

AMP concentrations, cell samples were taken 2 min after addition of the effectors for determining the cyclic AMP concentration in the cells plus medium and the activity of the cyclic AMP-dependent protein kinase. Glucagon was added as a positive control and, in agreement with previous studies in the literature (Cherrington *et al.*, 1976), increased both the formation of cyclic AMP by the cells and the activity ratio of the protein kinase (Table 3). In contrast with the effect of glucagon, Table 3 shows clearly that, despite significantly stimulating phosphorylase activity, neither [Leu]enkephalin nor dynorphin increased the concentration of cyclic AMP in the cells plus medium or altered the cyclic AMP-dependent protein kinase activity ratio, confirming that the mode of action of both of these peptides is different from that of β -endorphin (Matsumura *et al.*, 1984) and comparable with those of the catecholamines and Ca²⁺-dependent peptides, angiotensin and vasopressin. The similarity between the effects of the opioid peptides and those of the catecholamines and angiotensin at the level of the liver is further exemplified by the finding that both [Leu]enkephalin and dynorphin-(1–13) not only failed to stimulate cyclic AMP production but caused a significant decrease in the concentration of cyclic AMP within the cells, by 45% and 40% on addition of [Leu]enkephalin and dynorphin-(1–13) respectively (Table 3). It is now

accepted that angiotensin and α_2 -adrenergic agonists, but not vasopressin, are capable of inhibiting adenylate cyclase in liver (Jard *et al.*, 1981; Crane *et al.*, 1982) and therefore the possibility exists that the opioid peptides may also act in a similar manner. This would be further evidence that discrete opiate receptors may be involved in the response in the liver, as it is well established that opioid peptides can inhibit adenylate cyclase activity in neuronal tissue, particularly the neuroblastoma $\times$ glioma hybrid cell line NG108-15 (reviewed by Klee *et al.*, 1984). Before this can be established, measurements of the effects of opioid peptides on adenylate cyclase activity in purified liver plasma membranes must be undertaken, since it is evident that vasopressin and angiotensin may also lower cyclic AMP concentrations in isolated hepatocytes by activating phosphodiesterase activity (Keppens & De Wulf, 1984).

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