Changes in free cytosolic calcium and accumulation of inositol phosphates in isolated hepatocytes by [Leu]enkephalin

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Isolated hepatocytes from fed rats were used to study the effects of the opioid peptide [Leu]enkephalin on intracellular free cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$) and inositol phosphate production. By measuring the fluorescence of the intracellular Ca^{2+} -selective indicator quin-2, [Leu]enkephalin was found to increase $[\text{Ca}^{2+}]_i$ rapidly from a resting value of $0.219 \mu\text{M}$ to $0.55 \mu\text{M}$. The magnitude of this response was comparable with that produced by maximally stimulating concentrations of either vasopressin (100 nM) or phenylephrine (10 μM). The opioid-peptide-mediated increase in $[\text{Ca}^{2+}]_i$ showed a dose-dependency comparable with the activation of phosphorylase, but it preceded the increase in phosphorylase a activity. Addition of [Leu]enkephalin to hepatocytes prelabelled with *myo*-[2- ^3H (n)]inositol resulted in a significant stimulation of inositol phosphate production. At 10 min after hormone addition, there were increases in the concentrations of inositol mono-, bis- and tris-phosphate fractions of 12-, 9- and 14-fold respectively. No effect was apparent on the glycerophosphoinositol fraction. The effect of 10 μM -[Leu]enkephalin on inositol phosphate production was significantly greater than that obtained with 10 μM -phenylephrine, but marginally smaller than that induced by 100 nM-vasopressin. However, at these concentrations all three agonists gave a comparable increase in $[\text{Ca}^{2+}]_i$ and activation of phosphorylase a . These data provide evidence for [Leu]enkephalin acting via a mechanism involving a mobilization of Ca^{2+} as a result of increased phosphatidylinositol turnover.

INTRODUCTION

It is generally accepted that catecholamines, vasopressin and angiotensin stimulate glycogenolysis and gluconeogenesis in isolated hepatocytes by a Ca^{2+} -dependent mechanism (for reviews, see Exton, 1981; Williamson *et al.*, 1981). The fluorescent intracellular Ca^{2+} -indicator quin-2 has been used to measure the dose-dependency and time of onset of the increase in the cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) after cell stimulation. The data were consistent with the role of Ca^{2+} as an intracellular messenger in the activation of glycogenolysis and increase in phosphorylase activity (Charest *et al.*, 1983, 1985; Berthon *et al.*, 1984; Thomas *et al.*, 1984). In hepatocytes, the initial rise in $[\text{Ca}^{2+}]_i$ appears to be a consequence of the release of Ca^{2+} from intracellular stores, probably a component of the endoplasmic reticulum, with an influx of Ca^{2+} from the external medium being required to augment the maximal response obtained and to sustain the elevated $[\text{Ca}^{2+}]_i$ (Charest *et al.*, 1985; Binet *et al.*, 1985). It has been proposed that the breakdown of inositol phospholipids, particularly phosphatidylinositol 4,5-bisphosphate, is the transducing mechanism responsible for coupling receptor occupation at the plasma membrane to the mobilization of intracellular Ca^{2+} stores (Kirk *et al.*, 1981; Michell *et al.*, 1981; Berridge *et al.*, 1983; Creba *et al.*, 1983; Litosch *et al.*, 1983; Thomas *et al.*, 1984). The water-soluble product of this reaction, inositol 1,4,5-trisphosphate, is thought to act as a second messenger which releases Ca^{2+} from the intracellular hormone-sensitive Ca^{2+} pool (Streb *et al.*, 1983; Berridge *et al.*, 1984; Burgess *et al.*, 1984a,b; Joseph *et al.*, 1984).

In previous studies, we have shown that challenging hepatocytes with [Leu]enkephalin results in a stimulation of glycogenolysis and gluconeogenesis through a cyclic AMP-independent mechanism (Allan *et al.*, 1983; Leach *et al.*, 1985; Leach & Titheradge, 1986). The effect is dependent on the availability of extracellular Ca^{2+} , and therefore it has been suggested that the opioid peptides act in the liver by a similar mechanism to that employed by the vasoactive peptides angiotensin and vasopressin (Leach & Titheradge, 1986). To investigate this possibility, the effect of [Leu]enkephalin on Ca^{2+} mobilization and inositol phosphate production has been examined.

EXPERIMENTAL

Materials

[Leu]enkephalin, phenylephrine, vasopressin, collagenase, quin-2/AM, DTPA, bacitracin and digitonin were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). [^{14}C]Glucose 1-phosphate and L-[^{14}C]inositol 1-phosphate were obtained from Amersham International (Amersham, Bucks., U.K.). *myo*-[2- ^3H (n)]inositol was obtained from DuPont (NEN) Research Products (Boston, MA, U.S.A.). Before use, this was dried under N_2 and passed through a 1 cm column of Dowex 1 (X10; 100–200 mesh; formate form) to remove contaminants. Dowex 1 (X10, 100–200 mesh, Cl^-) ion-exchange resin was from Fluka A.G. (Buchs, Switzerland). ^{32}P -labelled inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate were prepared as described by Downes & Michell (1981). All other chemicals either were of AnalaR grade from BDH Chemicals (Poole, Dorset, U.K.) or were from Sigma.

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular free cytosolic Ca^{2+} concentration; DTPA, diethylenetriaminepenta-acetic acid.

Fig. 1. Measurement of intracellular Ca^{2+} mobilization by using quin-2

Hepatocytes were prepared and loaded with quin-2 as described in the Experimental section. The fluorescence was calibrated after the addition of $10 \mu\text{M}$ -[Leu]enkephalin by the sequential addition of $20 \mu\text{M}$ -digitonin and $100 \mu\text{M}$ -DTPA, followed by 2 mM -EGTA. The fluorescence changes in control cells are shown by the broken line.

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 final incubation volume and the incubation period with peptides or hormones 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 were removed at the times stated in the text.

Phosphorylase *a* activity was determined as in Leach *et al.* (1985). As described previously (Charest *et al.*,

1983; Thomas *et al.*, 1984), quin-2 loading and dimethyl sulphoxide treatment of hepatocytes increased the basal activity of phosphorylase *a* in the cells by 56%, and therefore, in experiments in which phosphorylase *a* activity was compared with Ca^{2+} mobilization, the enzyme was measured in cells incubated under identical conditions but in the absence of quin-2 and dimethyl sulphoxide.

$[\text{Ca}^{2+}]_i$ was measured by using quin-2 by the method of Charest *et al.* (1985) as modified by Sistare *et al.* (1985). Cells were resuspended at 50 mg wet wt./ml in modified Krebs–Henseleit bicarbonate buffer containing 5 mM - Ca^{2+} , 20 mM -Hepes and 1% fatty-acid-free bovine serum albumin (fraction V) and preincubated for 15 min. Half of the cells were loaded with quin-2/AM by the addition of the indicator in dimethyl sulphoxide to a final concentration of $100 \mu\text{M}$, followed by a 20 min incubation period. The remaining cells received an equivalent amount of dimethyl sulphoxide (final concn. 0.2%, v/v). After the loading period, both groups of cells were collected by gentle centrifugation (1 min at 500 g) and resuspended at 25–35 mg wet wt./ml. Each of the cell suspensions was divided into 3.5 ml batches in 25 ml flasks, gassed with O_2/CO_2 (19:1) and stored on ice until required. For the determination of quin-2 fluorescence, the cells were incubated for 10 min at 37°C before being transferred to a cuvette in a Perkin–Elmer fluorescence spectrometer. The cells were maintained at 37°C , constantly stirred and gassed continually over the surface with O_2/CO_2 (19:1). Fluorescence of the cells was determined at an excitation wavelength of 340 nm (5 nm slit-width) and an emission wavelength of 500 nm (slit-width 20 nm). In a typical experiment, vehicle or hormone was added to the cells after the fluorescence trace had settled and the fluorescence (*F*) was determined. On completion of the fluorescence measurements, the cells were permeabilized with $20 \mu\text{M}$ -digitonin (final concn.) in the presence of $100 \mu\text{M}$ -DTPA (Rink & Pozzan, 1985) to determine the maximum fluorescence of the Ca^{2+} -saturated quin-2 (F_{max}) and then treated with 2 mM -EGTA in 25 mM -Tris, pH 8.3, to determine the minimum fluorescence of the quin-2 in the absence of free Ca^{2+} (F_{min}). Control incubations were carried out with non-loaded cells to determine the contribution to the fluorescence made by changes in the redox state of the nicotinamide nucleotides on treatment of the cells, and the quin-2 fluorescence was corrected for this. The $[\text{Ca}^{2+}]_i$ before and after hormone addition was calculated from the corrected values by the equation

$$[\text{Ca}^{2+}]_i = K_D(F - F_{\text{min}})/(F_{\text{max}} - F)$$

(Tsien *et al.*, 1982).

Inositol phosphate production in the cells was determined essentially as described previously (Downes & Michell, 1981; Bone *et al.*, 1984). The hepatocytes were suspended at approx. 50 mg wet wt./ml and incubated in Krebs–Henseleit bicarbonate buffer containing 2% (w/v) bovine serum albumin (fraction V), 10 mM -glucose and $0.6 \mu\text{M}$ -*myo*-[2- ^3H (n)]inositol (sp. radioactivity 16.5 Ci/mmol). The cells were gassed with O_2/CO_2 (19:1) every 15 min and the preincubation period was continued for 90 min. At the end of the preincubation period, the [^3H]inositol was diluted with unlabelled inositol (5 mM final concn.) and 10 mM -LiCl was added to minimize the hydrolysis of any inositol phosphates formed during cell stimulation. Cells were then divided

Table 1. Effect of maximal concentrations of [Leu]enkephalin, vasopressin and phenylephrine on $[\text{Ca}^{2+}]_i$

Hepatocytes were loaded with quin-2 and $[\text{Ca}^{2+}]_i$ determined after the maximal change in fluorescence observed after addition of the effectors as described in the Experimental section. 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.

Additions	$[\text{Ca}^{2+}]_i$ (μM)
Control	0.22 ± 0.03
[Leu]enkephalin (10 μM)	$0.55 \pm 0.09^{**}$
Vasopressin (100 nM)	$0.59 \pm 0.09^{**}$
Phenylephrine (10 μM)	$0.54 \pm 0.09^{**}$

into batches in the presence of 0.1% (w/v) bacitracin and samples were quenched 5 and 10 min after hormone addition in 0.45 M-HClO₄ (final concn.). The extract was then vortex-mixed and the precipitate removed by centrifugation in an Eppendorf micro-centrifuge at 10000 *g* for 15 s before neutralization with 1.5 M-KOH containing 75 mM-Hepes. Samples were then frozen overnight, before anion-exchange chromatography for the separation of inositol phosphates. Parallel incubations were carried out in the absence of the labelled inositol for the determination of phosphorylase *a* activity. The water-soluble derivatives of the *myo*-[2-³H(n)]inositol were separated by loading 0.5 ml of the neutralized cell extracts in 10 ml of water on to 7 cm columns of Dowex 1 (X10; 100–200 mesh; formate form) prepared as described by Creba *et al.* (1983). The columns were sequentially eluted with 20 ml of water to remove free inositol, 20 ml of 60 mM-ammonium formate to remove glycerophosphoinositol ('GroPIns fraction'), 20 ml of 180 mM-ammonium formate to remove inositol monophosphate ('InsP fraction'), 30 ml of 400 mM-ammonium formate/0.1 M-formic acid to remove inositol bisphos-

phate ('InsP₂ fraction') and 20 ml of 1.0 M-ammonium formate/0.1 M-formic acid to remove inositol trisphosphates ('InsP₃ fraction'). The radioactivity in 2 ml samples of each of these fractions was determined by liquid-scintillation counting. The use of anion-exchange columns for these separations was validated by using radiolabelled samples of the individual inositol phosphates. It was noted that up to 10% of the inositol 1,4-bisphosphate was eluted in the 'InsP₃ fraction'; this was not accounted for in the calculations; however, there was no significant contamination of the 'InsP₂ fraction' with inositol trisphosphate.

Results are expressed as means $\pm$ S.E.M. Statistical analysis of the results was performed by using a pooled *t*-test.

RESULTS AND DISCUSSION

Effect of [Leu]enkephalin on $[\text{Ca}^{2+}]_i$

Previous studies have suggested that the effects of the enkephalins and dynorphin-(1–13) on hepatic carbohydrate metabolism are mediated by a Ca^{2+} -dependent mechanism (Allan *et al.*, 1983; Leach *et al.*, 1985; Leach & Titheradge, 1986). We have therefore examined the effect of addition of [Leu]enkephalin to isolated hepatocytes on the mobilization of intracellular Ca^{2+} , as judged by the increase in fluorescence of the Ca^{2+} chelator quin-2. Fig. 1 shows that after addition of 10 μM -[Leu]enkephalin there was a rapid rise in $[\text{Ca}^{2+}]_i$, as indicated by the increased quin-2 fluorescence. Also shown in Fig. 1 is the effect of [Leu]enkephalin on the fluorescence of control cells not loaded with quin-2. In agreement with previous studies using catecholamines and glucagon (Charest *et al.*, 1983; Sistare *et al.*, 1985), there was a small but significant increase in fluorescence in the absence of quin-2, which accounted for approx. 20% of the total signal. This has been accounted for by a shift in the nicotinamide nucleotides to a more reduced state on stimulation of the cells by effectors (Charest

Fig. 2. Sensitivity of the cells to [Leu]enkephalin concentration as shown by $[\text{Ca}^{2+}]_i$

Hepatocytes were prepared and loaded with quin-2 as described in the Experimental section. After the fluorescence trace had stabilized, [Leu]enkephalin was added to give the final concentrations indicated below each trace.

Fig. 3. Comparison of dose-response for [Leu]enkephalin on phosphorylase activation and $[Ca^{2+}]_i$.

$[Ca^{2+}]_i$ was determined in quin-2-loaded hepatocytes as described in the Experimental section. Phosphorylase *a* activity was determined 5 min after the addition of effector in cells incubated under identical conditions but in the absence of quin-2 and dimethyl sulphoxide. Results are expressed as means $\pm$ S.E.M. for three and four different cell preparations respectively.

et al., 1983) and has been allowed for in all calculations of the intracellular free Ca^{2+} concentration. The cytosolic concentration of resting free Ca^{2+} was calculated to be $0.219 \pm 0.027 \mu M$, which is in good agreement with the previously reported values for isolated hepatocytes obtained by this method ($0.16 \mu M$, Thomas *et al.*, 1984; $0.2 \mu M$, Charest *et al.*, 1983). The ability of a maximal concentration of [Leu]enkephalin ($10 \mu M$) to mobilize intracellular Ca^{2+} was compared with that of maximal doses of vasopressin (100 nM) and phenylephrine ($10 \mu M$), and the results are shown in Table 1. It is clear from Table 1 that $10 \mu M$ -[Leu]enkephalin is as efficacious at elevating $[Ca^{2+}]_i$ as maximally stimulating concentrations of phenylephrine and vasopressin. The degree of stimulation (250%) by the opioid peptide, vasopressin and phenylephrine agrees favourably with that reported by others using similar methods (Thomas *et al.*, 1984; Charest *et al.*, 1983).

Comparison of the dose-response of [Leu]enkephalin on $[Ca^{2+}]_i$ and phosphorylase activation

In order to demonstrate that the changes in $[Ca^{2+}]_i$ brought about by [Leu]enkephalin are responsible for the activation of phosphorylase and glycogenolysis, the effect of addition of increasing concentrations of the opioid peptide on quin-2 fluorescence and phosphorylase *a* activity was determined. Fig. 2 shows that [Leu]enkeph-

alin increased the quin-2 fluorescence over the entire range tested between 0.3 nM and $10 \mu M$. It is possible that lower doses of the opioid peptide may also increase $[Ca^{2+}]_i$ and phosphorylase *a* activity; however, the extremely rapid degradation of the enkephalin by hepatocytes may prevent detection (Leach *et al.*, 1985). The time required to achieve a maximal fluorescence was also dependent on the concentration of opioid peptide, with lower concentrations of [Leu]enkephalin producing a slower rise in $[Ca^{2+}]_i$. A similar effect has been reported after glucagon treatment of cells (Sistare *et al.*, 1985). Fig. 3 shows a comparison of the mobilization of intracellular Ca^{2+} and phosphorylase *a* activity with increasing concentrations of the opioid peptide. It is evident that both parameters responded to the opioid peptide over a similar range of concentrations. The half-maximal concentration of [Leu]enkephalin required was $0.96 \pm 0.32 \mu M$ (4) and $0.56 \pm 0.23 \mu M$ (3) for phosphorylase activation and Ca^{2+} mobilization respectively ($P > 0.05$). The concentration of effector required for a maximal stimulation was also comparable, being $10 \mu M$ -[Leu]enkephalin in both cases. The characteristics of the change in $[Ca^{2+}]_i$ are consistent with the role of Ca^{2+} as an intracellular messenger during the opioid-peptide-mediated breakdown of glycogen.

Time course for the [Leu]enkephalin-mediated increase in $[Ca^{2+}]_i$ and phosphorylase activation

We have previously postulated that the effect of the enkephalins and dynorphin-(1-13) on glycogenolysis is the result of a Ca^{2+} -dependent activation of phosphorylase *a*, presumably mediated through phosphorylase kinase (Leach *et al.*, 1985; Leach & Titheradge, 1986). The time of onset for the increase in phosphorylase *a* and $[Ca^{2+}]_i$ after treatment of the cells with a maximal

Fig. 4. Time course for phosphorylase activation and mobilization of $[Ca^{2+}]_i$ by [Leu]enkephalin

$[Ca^{2+}]_i$ ($\square$) was determined in quin-2-loaded hepatocytes as described in the Experimental section. Phosphorylase *a* activity ($\circ$) was determined after the addition of effector at the times indicated. Results are expressed as means $\pm$ S.E.M. for four different cell preparations.

Table 2. Effect of maximal concentrations of [Leu]enkephalin, vasopressin and phenylephrine on hepatocyte inositol metabolism

The water-soluble derivatives of the *myo*-[2- ^3H (n)]inositol were measured 5 and 10 min after addition of the effectors by chromatography on Dowex ion-exchange columns as described in the Experimental section. Phosphorylase *a* activity was determined in control cells incubated under the same conditions and is expressed in terms of $\mu\text{mol}/\text{min}$ per g dry wt. Results are expressed as means $\pm$ S.E.M. for four different cell preparations. * $P < 0.05$ and ** $P < 0.005$ for difference from appropriate controls.

Additions	Fraction . . .	$10^{-5} \times$ Radioactivity recovered (d.p.m./g dry wt.)				Phosphorylase activity
		GroPIIns	InsP	InsP ₂	InsP ₃	
Control ($t = 5$ min)		21.44 $\pm$ 2.36	5.27 $\pm$ 0.33	2.09 $\pm$ 0.72	0.22 $\pm$ 0.09	40.53 $\pm$ 3.58
[Leu]enkephalin (10 μM)		21.62 $\pm$ 4.33	32.51 $\pm$ 4.36**	13.15 $\pm$ 2.81*	3.37 $\pm$ 0.27**	80.10 $\pm$ 3.75**
Vasopressin (100 nM)		18.85 $\pm$ 1.88	36.94 $\pm$ 4.50**	27.34 $\pm$ 4.40**	5.39 $\pm$ 0.22**	86.60 $\pm$ 5.10**
Phenylephrine (10 μM)		18.42 $\pm$ 2.57	9.65 $\pm$ 0.52**	2.76 $\pm$ 0.28	0.78 $\pm$ 0.11*	72.05 $\pm$ 3.27**
Control ($t = 10$ min)		20.06 $\pm$ 2.41	4.55 $\pm$ 1.38	1.32 $\pm$ 2.95	0.30 $\pm$ 0.12	
[Leu]enkephalin (10 μM)		20.89 $\pm$ 2.66	54.78 $\pm$ 6.81**	12.18 $\pm$ 2.66*	4.29 $\pm$ 0.30**	
Vasopressin (100 nM)		21.20 $\pm$ 3.12	72.15 $\pm$ 7.46**	37.43 $\pm$ 5.63**	6.20 $\pm$ 0.49**	
Phenylephrine (10 μM)		19.29 $\pm$ 3.12	14.71 $\pm$ 1.42**	2.84 $\pm$ 0.54	1.06 $\pm$ 0.17*	

concentration of [Leu]enkephalin is shown in Fig. 4. On addition of the opioid peptide to the cells, there was a rapid 4-fold increase in the intracellular concentration of Ca^{2+} within 15–30 s. This was a transient rise and, even in the presence of this high concentration of effector, the response was not sustained and by 2 min had declined by 50%. There was no change in $[\text{Ca}^{2+}]_i$ in control cells over this time period. The time course for the mobilization of $[\text{Ca}^{2+}]_i$ by [Leu]enkephalin was comparable with that reported after addition of 10 nM-vasopressin, but slower in onset than that found after stimulation of the cells by either 1 μM -adrenaline or 10 μM -phenylephrine (Charest *et al.*, 1983). The activation of phosphorylase by [Leu]enkephalin showed a significant delay when compared with Ca^{2+} mobilization. Although there was a significant increase in the amount of phosphorylase *a* within 15 s, the maximal activity was not achieved until 60–120 s after the addition of the peptide. In contrast with the quin-2 fluorescence, the phosphorylase response to the opioid peptide was not as transient and was maintained for 5 min before slowly returning to control values (results not shown).

The refractoriness of the cells to [Leu]enkephalin noted previously with glycogen mobilization (Leach & Titheradge, 1986) was clearly evident in quin-2-loaded hepatocytes, in that, after stimulation by [Leu]enkephalin, a further addition of the opioid peptide, phenylephrine or vasopressin failed to increase $[\text{Ca}^{2+}]_i$, even when the cytosolic concentration of Ca^{2+} had returned to resting values, unless a sub-maximal dose of the enkephalin was used (results not shown).

Effect of [Leu]enkephalin on inositol phosphate production

Table 2 shows the effect of addition of 10 μM -[Leu]enkephalin to cells prelabelled with *myo*-[2- ^3H (n)]-inositol on the production of the water-soluble inositol phosphates. The experiments were carried out in the presence of 10 mM-LiCl to increase the accumulation of the inositol phosphates (Thomas *et al.*, 1984; Berridge *et al.*, 1984). Vasopressin and phenylephrine were used as positive controls. All three effectors significantly stimulated the accumulation of the InsP, InsP₂ and InsP₃ fractions, but not that of the GroPIIns fraction; however,

the magnitude of the stimulation varied considerably with the agonist used. After a 5 min incubation period with the effector, [Leu]enkephalin increased the radioactivity of the InsP, InsP₂ and InsP₃ fractions by 6.2-, 6.3- and 15.3-fold respectively. With the exception of the InsP₃ fraction, the degree of the accumulation was further enhanced by increasing the incubation period to 10 min. As described previously, the effect of α -adrenergic agents on InsP₃ formation was considerably less than that of vasopressin, although these agents produced a comparable increase in both phosphorylase *a* activity (Table 2) and $[\text{Ca}^{2+}]_i$ (Table 1) (Charest *et al.*, 1985; Lynch *et al.*, 1985). The difference in magnitude of the response elicited by vasopressin and phenylephrine was apparent on the accumulation of all of the inositol phosphates and was significant at both times measured ($P < 0.01$). The response of the cells to [Leu]enkephalin was intermediate between that to vasopressin and phenylephrine, with the formation of the inositol phosphates being significantly greater than that induced by the catecholamines ($P < 0.02$). These observations support our previous proposal (Leach *et al.*, 1985) that enkephalins act on the liver by a mechanism which is similar to that of vasopressin and angiotensin. It is clear that all three agonists mobilize intracellular Ca^{2+} as a result of receptor occupancy and inositol lipid turnover. The apparent discrepancy between the magnitude of the response obtained for the accumulation of inositol phosphates, compared with Ca^{2+} mobilization and phosphorylase activation, may have several explanations. First, Lynch *et al.* (1985) have demonstrated that the formation of inositol trisphosphate correlates closely with the maximum receptor-binding capacity of the cells for the respective hormones, whereas the activation of phosphorylase and mobilization of Ca^{2+} occur in response to a small sub-maximal elevation of inositol lipid turnover. Second, it is evident that the InsP₃ fraction from hepatocytes which is measured in this type of separation procedure consists not only of inositol 1,4,5-trisphosphate but also of the 1,3,4 isomer (Burgess *et al.*, 1984a,b, 1985). As the rate of formation of these two isomers is very different (Irvine *et al.*, 1984, 1985; Burgess *et al.*, 1985) and it may only be the 1,4,5 isomer which mobilizes Ca^{2+} from intracellular stores (Streb

et al., 1983; Berridge *et al.*, 1984; Burgess *et al.*, 1984a,b; Joseph *et al.*, 1984), this may also account, at least in part, for the discrepancy.

From these experiments it is evident that [Leu]-enkephalin can now be added to the growing list of agonists known to cause inositol lipid turnover and Ca^{2+} mobilization in isolated hepatocytes. The receptors responsible for these effects still remain to be identified; however, our previous studies would suggest that, if classical opiate receptors are involved, then they are of the kappa- or delta-type, as [Leu]enkephalin is thought to act predominantly via delta-receptors, whereas the effects of dynorphin are believed to be via kappa-receptors (reviewed by Cox, 1982). It seems unlikely that the mu-receptor is involved, as morphine was inactive and naloxone, the mu-receptor antagonist, showed no or only a weak antagonism of the effect of [Leu]enkephalin on carbohydrate metabolism (Allan *et al.*, 1983). The physiological role of this effect of opioid peptides still remains to be established, as it is evident that the concentration of the peptide required to elicit the response is considerably higher than the plasma concentration found even under stress conditions (Frederickson & Geary, 1982). This apparent lack of sensitivity may be the result of the very rapid degradation of the [Leu]enkephalin when incubated under static conditions with isolated hepatocytes (Leach *et al.*, 1985). Alternatively, the sensitivity of the receptors may reflect the local concentrations of opioid peptides achieved in the liver as a result of release from the liver itself or sympathetic innervation. In favour of this, work by Wadja *et al.* (1976) has demonstrated that substances with opiate agonist-like activity are present in the liver, as demonstrated by their ability to displace labelled opiate ligands from brain membranes. These two possibilities require investigation.

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