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The occurrence and receptor specificity of endogenous opioid peptides within the pancreas and liver of the rat

Comparison with brain

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Our observations that opioid peptides have direct effects on islet insulin secretion and liver glucose production prompted a search for endogenous opiates and their receptors in these peripheral tissues. μ-, δ- and κ-receptor-active opiates were demonstrated in brain, pancreas and liver extracts by displacement studies using selective ligands for the three opiate receptor subtypes ([^{3}H][D-Ala^2,MePhe^4,Gly^5-ol]enkephalin, [^{3}H][D-Ala^2,D-Leu^5]enkephalin and [^{3}H]dynorphin respectively). Receptor-active opiates in brain extracts exhibited a stronger preference for δ-opiate-receptor sites than for μ and κ sites. Pancreatic extract opiates demonstrated a similar activity at μ and δ sites, but substantially less at κ sites. Liver extracts displayed similar selectivity for all three sites. The affinities of the receptor-active opiates for μ-, δ- and κ-receptor subtypes displayed a rank order of potency: brain $\gg$ pancreas $>$ liver. Total immunoreactive β-endorphin and [Met^5]enkephalin levels in liver and hepatocytes were greater than those in brain. Immunoreactive [Met^5]enkephalin levels in pancreas were similar to, but β-endorphin levels were substantially higher than, those in brain. δ and κ opiate-binding sites of high affinity were identified in crude membrane preparations of islets of Langerhans, but no specific opiate-binding sites could be demonstrated in liver membrane preparations. Immunoreactive dynorphin and β-endorphin were demonstrated by immunogold labelling in rat pancreatic islet cells. No positive staining of liver sections for opioids was observed. These results suggest that the tissue content of opiate-receptor-active compounds in the pancreas and the liver is very significant and could contribute to the regulation of normal blood glucose levels.

INTRODUCTION

A centrally mediated sympathetic connection between opioid peptides and glucose homoeostasis has been established for some time (Bodo *et al.*, 1937; Vassalle, 1961; Feldberg & Smyth, 1977; Pfeiffer *et al.*, 1983); however, evidence for a direct peripheral action of opiates in the regulation of hepatic glucose production and on glucose homoeostasis through pancreatic-islet hormone secretion has only more recently been reported (Ipp *et al.*, 1978; Green *et al.*, 1980, 1983*a*,*b*; Allan *et al.*, 1983; Leach *et al.*, 1985; Leach & Titheradge, 1986; Hothi *et al.*, 1988). β-Endorphin, [Met^5]enkephalin, [Leu^5]enkephalin and dynorphin A-(1–13)-peptide have been shown to regulate insulin, glucagon and somatostatin release from isolated islets of Langerhans by a naloxone-reversible mechanism (Ipp *et al.*, 1978; Green *et al.*, 1980, 1983*a*,*b*; Hermansen, 1983; Curry *et al.*, 1987; Toyota *et al.*, 1985). Similarly, a number of studies have demonstrated that these opioid peptides are localized within the pancreas in a variety of species. Acid extracts prepared from pancreatic tissues of human (Feurle *et al.*, 1982) and guinea pig (Stern *et al.*, 1982) revealed immunoreactive [Met^5]enkephalin and [Leu^5]enkephalin as well as [Met^5]enkephalin-Arg^6-Phe^7 (MERF) and [Met^5]enkephalin-Arg^6-Gly^7-Leu^8 (MERGL). The presence of immunoreactive β-endorphin has been described in pancreatic extracts of rat (Smyth & Zakarian, 1982) and pig (Houck *et al.*, 1981), and low-molecular-mass forms of β-endorphin have been found in humans (Bruni *et al.*, 1979). The presence of dynorphin-like factors ([Leu^5]enkephalin-Arg^6-containing sequences) has been shown in extracts of guinea-pig pancreas (Stern *et al.*, 1982). Attempts to identify the opiate receptors involved in pancreas have been few; however, Barkey *et al.* (1981) detected high-affinity μ opiate-binding sites in crude pancreatic membrane preparations, and Verspohl *et al.* (1986) described δ opiate-binding sites in isolated islets of Langerhans. These published reports have not been confirmed, and no attempt has been made to measure κ-opiate-receptor binding, despite the fact that dynorphin can profoundly influence hormone secretion.

Direct effects of opioid peptides on glucose mobilization in isolated hepatocytes have also been described. β-Endorphin (Matsumura *et al.*, 1984), [Met^5]enkephalin, [Leu^5]enkephalin and dynorphin A-(1–13)-peptide (Allan *et al.*, 1983; Leach *et al.*, 1985; Leach & Titheradge, 1986) have all been shown to induce a rapid dose-dependent stimulation of both glycogenolysis and glycogenesis. The mechanism of action of β-endorphin has been attributed to a rise in cyclic AMP (Matsumura *et al.*, 1984), whereas the enkephalins and dynorphin decrease cyclic AMP levels and adenylate cyclase activity and increase inositol lipid turnover and cytosolic Ca^{2+} (Leach & Titheradge, 1986; Leach *et al.*, 1986; Hothi *et al.*, 1988). Wajda *et al.* (1976) have identified the existence of an opiate-like factor in Tris/HCl extracts of rat liver by its ability to displace [^{3}H]naloxone and [^{3}H]dihydromorphine from rat brain membrane receptors in a dose-dependent fashion. However, the morphine-like activity, measured by radioreceptor binding assays, had little or no naloxone-reversible action on the electrically stimulated guinea-pig ileum (Wajda *et al.*, 1976). This has not been characterized further. Attempts to measure specific receptors for opiates in liver have suggested that two specific binding sites for β-endorphin may exist in hepatic plasma membranes, at least one of which was linked to adenylate cyclase (Dave *et al.*, 1985). No studies have been published

Abbreviations used: DAGO, [D-Ala^2,MePhe^4,Gly^5-ol]enkephalin; deltakephalin, [D-Thr^2,Leu^5]enkephalin-D-Thr^6; DHM, dihydromorphine; DADL, [D-Ala^2,D-Leu^5]enkephalin; DYN(1–8), dynorphin A-(1–8)-peptide; DYN(1–9), dynorphin A-(1–9)-peptide; DPN, [^{3}H]diprenorphine; MERF, [Met^5]enkephalin-Arg^6-Phe^7; MERGL, [Met^5]enkephalin-Arg^6-Gly^7-Leu^8; PBS, phosphate-buffered saline (4 mM-sodium phosphate/150 mM-NaCl, pH 7.5); IC_{50}, concentration of extract required to give 50% displacement of the labelled agonists.

describing receptors for other opioid peptides; however, recent work has suggested that the effect of the enkephalins at least may be explained as a result of cross-reactivity of the peptide with angiotensin II receptors (Hothi *et al.*, 1989).

The aim of the present study was to investigate the occurrence of 'endogenous opioid peptides' in extracts of rat pancreas and liver and to characterize and localize these peptides by radioreceptor assay, radioimmunoassay and immunocytochemistry. This would determine whether they play a role in the peripheral regulation of glucose homoeostasis. The presence of specific opiate-binding sites in pancreas was also investigated to confirm the existence of μ and δ opiate receptors and explore the possibility that κ-opiate receptors may also be present. Similarly, the presence of specific opiate receptors in the liver was examined to determine whether the endogenous opiates may have a direct action *in vivo*, and whether the effects observed *in vitro* may be the result of cross-reactivity at other non-opiate receptors.

EXPERIMENTAL

Materials

[^{3}H]Diprenorphine, [^{3}H]dihydromorphine, [^{3}H][D-Ala^2,D-Leu^5]enkephalin, [^{3}H]dynorphin A-(1–8)-peptide and [^{3}H]-dynorphin A-(1–9)-peptide were obtained from Amersham International (Amersham, Bucks., U.K.). DAGO ([D-Ala^2, MePhe^4,Gly^5-ol]enkephalin), deltakephalin ([D-Thr^2,Leu^5]enkephalin-D-Thr^6, dynorphin A-(1–13)-peptide and human β-endorphin were obtained from Bachem AG (Bubendorf, Switzerland). Naloxone, [Leu^5]enkephalin, [Met^5]enkephalin, bacitracin, bestatin, L-leucyl-L-leucine and collagenase type V were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Trasylol was obtained from Bayer Pharmaceuticals (Haywards Heath, West Sussex, U.K.). Captopril was from Squibb and Sons (Wirral, Merseyside, U.K.). Tris was from Boehringer Corp. Ltd. (Lewes, East Sussex, U.K.). All other chemicals were either of AnalaR grade from BDH Chemicals (Poole, Dorset, U.K.) or from Sigma. Sep-Pak C_{18} cartridges (octadodecasilyl-silica columns) were purchased from Sep-Pak, Waters Associate (Milford, MA., U.S.A. Glucagon mouse monoclonal antiserum Novoclone was a gift from Dr A. Moody, Novo (Copenhagen, Denmark). Anti-β-endorphin antiserum, raised in rabbits by using porcine β-endorphin, was from Amersham International. It cross-reacts also with β-lipotropin. [Leu^5]Enkephalin antiserum, raised in rabbits, was purchased from Peninsula Laboratories (St Helens, Merseyside, U.K.). It has 2.5% cross-reactivity with [Met^5]enkephalin. Anti-dynorphin antiserum, raised in rabbit (supplied by Professor L. Rees and Dr T. Howlett at St Bartholomew's Hospital, London EC1, U.K.), cross-reacts with dynorphin A-(1–17)-peptide, A-(1–13)-peptide, A-(1–12)-peptide, A-(1–11)-peptide, A-(1–10)-peptide, and has 30% cross-reactivity with dynorphin A-(1–9)-peptide and 5–10% cross-reactivity with dynorphin A-(1–8)-peptide. Normal non-immune sera from rabbit and goat were obtained from Miles Scientific (Slough, Berks, U.K.). LR Gold resin was from London Resin Co. (Basingstoke, Hants., U.K.). Protein A-gold and goat anti-rabbit gold-conjugated second-antibody probes were purchased and prepared from 10 nm and 15 nm colloidal gold as described in the Janssen Life Sciences Products handbook (1988) and stored at 4 °C.

Animals

Fed male and female Sprague–Dawley rats, weighing between 200 and 250 g, were raised on regular rat chow and tap water *ad lib.* They were maintained in a 12 h-dark/12 h-light cycle (beginning at 07.00 h) and in a temperature-controlled room (25 °C ± 2 °C).

Methods

Membrane fraction preparations. Rats were killed by decapitation and the brains (minus cerebella) were rapidly removed. Each brain was homogenized in 10 vol. of ice-cold Tris/HCl assay buffer (50 mM, pH 8.0 at 21 °C). The homogenate was centrifuged at 40000 *g* for 20 min at 4 °C. The membrane pellet was resuspended and incubated at 37 °C for 40 min in the presence of a cocktail of proteinase inhibitors [18 μM-bestatin, 224 μM-L-leucyl-L-leucine, Trasylol (200 kallikrein-inhibitory units/ml), bacitracin (150 units/ml) and 92 μM-captopril] to dissociate endogenously bound opioid peptide (Simantov *et al.*, 1976) while minimizing possible receptor degradation. After an additional centrifugation step, as described above, brain membranes were resuspended in assay buffer and stored at −70 °C until required.

Purified plasma membranes from rat liver were prepared essentially by the method of Neville (1968), as modified by Goldstein & Blecher (1976). The plasma membranes were suspended in Tris/HCl assay buffer to a final protein concentration of 20 mg/ml and stored at −70 °C.

Islet membranes were prepared from 5000–6000 islets isolated by collagenase digestion of rat pancreas (Howell & Taylor, 1968). The islets were homogenized in ice-cold Tris/HCl assay buffer and the suspension was incubated for 40 min at 37 °C with the addition of the proteinase inhibitors described above and then centrifuged at 40000 *g* for 20 min at 4 °C. The pellet containing a crude membrane preparation was resuspended in assay buffer to a final protein concentration of 1 mg/ml and was stored at −70 °C.

Measurement of opiate-binding sites. Preliminary experiments were carried out to provide information on the kinetics, temperature-dependence and stability of the radioligands employed (Khawaja, 1987). The presence of specific opiate receptors in the different membrane preparations was examined by the binding of the μ-, δ- and κ-receptor-selective tritiated opiates, dihydromorphine (DHM), [D-Ala^2,D-Leu^5]enkephalin (DADL) and dynorphin A-(1–8)-peptide [DYN(1–8)] respectively. In some experiments, the antagonist [^{3}H]diprenorphine (DPN) was used as a non-discriminative opiate-receptor ligand.

Binding experiments were carried out in Tris/HCl assay buffer containing approx. 0.2 mg of brain, islet or liver membrane protein. A 50 μl portion of tritiated radioligand [DHM, DADL and DYN(1–8)] was added to a final concentration of between 4 and 6 nM. Non-specific binding was determined by the addition to a parallel series of tubes of 50 μl of a 500-fold excess of DAGO, deltakephalin or dynorphin A-(1–13)-peptide respectively. The optimal incubation times were 3 h with [^{3}H]DHM and [^{3}H]DADL, and 20 min with [^{3}H]DYN(1–8) at 22 °C (Khawaja, 1987). For incubations with [^{3}H]DYN(1–8), a cocktail of proteinase inhibitors (see above) was added to the incubation medium; addition of inhibitors to the [^{3}H]DHM and [^{3}H]DADL assays did not improve the binding. Separation of free and membrane-bound radioligand was achieved by centrifugation at 10000 *g* for 5 min at 4 °C. The supernatant was decanted and the tips of the tubes, containing the pellet, were cut off, placed in β-vials and the membranes were solubilized in 0.1 M-NaOH overnight. A 4 ml portion of a toluene/Triton-X-100/2,5-diphenyloxazole (70:30:3, v/v/w)-based scintillation fluid was added and the radioactivity emitted was counted in a Beckman (LS1801) liquid scintillation counter.

Preparation and purification of tissue extracts. In order to prepare liver and hepatocyte extracts, perfusions were carried

out on pentobarbitol-anaesthetized rats. The abdominal wall was incised and the liver was perfused via the hepatic portal vein with Krebs–Ringer bicarbonate buffer (pH 7.4 at 37 °C) in a flow-through system to flush out blood contaminants. Hepatocytes were prepared by the method of Berry & Friend (1969) as described previously by Leach *et al.*, (1985), and were stored at −70 °C. Brain, pancreas and skeletal muscle were dissected out and immediately frozen in solid CO_2 and stored at −70 °C. Opiates were extracted from tissue samples by using a modified version of the method described by Pasternak *et al.* (1975). Briefly, tissue samples were rapidly thawed and homogenized in 10 vol. of 10 mM-Tris/HCl buffer in a silicone-treated Dounce homogenizer. Skeletal muscle was homogenized with a Brinkman Polytron homogenizer (setting no. 6 for 20–30 s). The homogenates were centrifuged at 100000 *g* for 1 h at 4 °C and the supernatants were kept on ice. The pellets remaining were resuspended in 2 vol. of Tris/HCl buffer and immersed in a boiling-water bath for 15 min. The boiled homogenates were centrifuged at 100000 *g* for 1 h at 4 °C and the clear supernatants from both centrifugation steps were combined.

Preliminary purification of the crude tissue extracts was discovered to be essential, and was achieved with reverse-phased Sep-Pak C_{18} cartridges. Each Sep-Pak cartridge was washed with 6 ml of acetonitrile, followed by 10 ml of distilled water, and pre-equilibrated with 10 ml of 0.1 % trifluoroacetic acid. The tissue extract was passed through the Sep-Pak cartridge. The cartridge was washed with a further 10 ml of 0.1 % trifluoroacetic acid and the opiate-like material was eluted with 6 ml of 50 % acetonitrile/0.1 % trifluoroacetic acid. The eluate was freeze-dried and the dried material was reconstituted in Tris/HCl assay buffer. Opioid peptide content was determined by radioreceptor assay and radioimmunoassay. The extraction recovery from the Sep-Pak purification step was 81 ± 5 % using [^{3}H]DADL (2–3 nM) and 77 ± 5 % using [^{3}H]DYN(1–9) (4–5 nM) (mean + S.E.M. for two experiments carried out in triplicate). Although some loss of opioid peptides was observed, this rapid and simple separation procedure facilitated the removal of non-opioid substances such as univalent and bivalent cations, which could interfere with opioid binding and affect the reliability of radioreceptor and radioimmunoassays. The results shown are not corrected for peptide loss.

Radioreceptor assay of tissue extracts. To each sample tube was added a 400 μl aliquot of rat brain homogenate (1 mg/ml), 50–450 μl aliquots of tissue extract, 50 μl of proteinase inhibitors (see above) and either 50 μl of [^{3}H]DPN or a selective opiate-receptor ligand [^{3}H]DHM, [^{3}H]DADL or [^{3}H[DYN(1–9) (1–2 nM final concns.). A parallel series of tubes contained 1 μM of respective unlabelled ligand to determine non-specific binding. The incubation volumes were adjusted to 1.0 ml with Tris/HCl assay buffer; '100 % specific binding' was defined as binding of labelled opiate to brain membranes minus non-specific binding, both determined in the absence of any tissue extract. Standard curves were constructed over a concentration range of 10^{-10}–10^{-6} M for DAGO, deltakephalin and dynorphin A-(1–13)-peptide by displacement of [^{3}H]DHM, [^{3}H]DADL and [^{3}H]DYN(1–9) respectively. Assays were performed at 4 °C for 7 h (DHM), 4.5 h (DADL) and 1.5 h [DYN(1–9)]. Separation of free and membrane-bound radioligand was achieved by rapid filtration through Whatman GF/B glass-fibre filter circles, pre-soaked with 1 % polyethyleneimine to minimize non-specific binding. Filters were washed with 12 ml of assay buffer and the radioactivity emitted was counted as described above. The protein concentration in all tissue membrane and Tris/HCl extract preparations was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin (fraction V) as the standard.

Opioid-peptide radioimmunoassays. β-Endorphin was radioimmunoassayed by Dr. J. Gomm (National Institute for Medical Research, Mill Hill, London NW7, U.K.). The (porcine) β-endorphin-directed antibody used cross-reacts with pro-opiomelanocortin and β-lipotropin (Zakarian & Smyth, 1982). Immunoreactive [Met^5]enkephalin, [Met^5]enkephalin-Arg^6-Phe^7 (MERF) and [Met^5]enkephalin-Arg^6-Gly^7-Leu^8 (MERGL) measurements were carried out by Dr S. Medbak (St Bartholomew's Hospital) according to the methods described by Clement-Jones *et al.* (1980) and Medbak *et al.* (1981).

Electron microscopy/immuno-gold labelling of islets. All the steps outlined below were carried out at 4 °C. Batches of 50–100 collagenase-isolated islets were picked directly into Eppendorf tubes and fixed in a mixture of 4 % formaldehyde and 0.5 % glutaraldehyde in phosphate-buffered saline (PBS) for 3 h. After thorough rinsing for 2 h in PBS buffer, the islets were dehydrated in an ethanol series and embedded in LR Gold resin. Over a period of 7–10 days, three or four changes of resin were made, the last change included 0.1 % benzil (a light-sensitive initiator for the resin). A polymerization time of 48 h was found to produce blocks of better sectioning quality than those produced at 24 h. Sections were picked up on 300-mesh nickel grids. All the incubation steps described were achieved by placing the grids on 10 μl droplets of solution. PBS buffer and double-glass-distilled-water rinses were made by floating the grids on filled wells of a 96-well microtitre tray. PBS containing 1 % bovine serum albumin, 1 mM-Na_2EDTA, 0.05 % Tween 20 and NaN_3 (0.2 mg/ml) (PBS+) was used throughout the procedure for rinsing and dilution of antisera. Initial blocking of non-specific antigen sites was carried out by pre-incubating the grids for 10 min with either PBS+ (in sections to be stained for glucagon and dynorphin) or 1 % normal goat serum in PBS+ (in sections to be stained for β-endorphin and normal rabbit serum). The sections were transferred to antisera directed against glucagon (1:200), dynorphin (1:80) and β-endorphin (1:80) and normal rabbit non-immune serum (1:80) for 2 h at room temperature or overnight at 4 °C in a humid box. Dynorphin antiserum was pre-incubated with glucagon to ensure that the dynorphin interaction with glucagon granules was specific. After three 2 min PBS+ rinses, sections were incubated in Protein A–gold or goat anti-rabbit gold for 1 h at room-temperature and rinsed in PBS+ (4 × 5 min) and double-glass-distilled water (4 × 10 min) before light post-staining in uranyl acetate (3 min). Sections were examined in a JEOL 100C electron microscope at 100 kV.

RESULTS AND DISCUSSION

Radioreceptor assay of tissue extracts

Fig. 1 shows displacement of [^{3}H]diprenorphine, a non-selective opiate-receptor antagonist, from rat brain membranes by Tris/HCl extracts of rat brain, pituitary, pancreas, liver, hepatocytes and skeletal muscle. It is evident that, with the exception of skeletal muscle, the extracts prepared from all of the other tissues contain opioid-like substances capable of competing in a dose-dependent manner with the radiolabelled opiate ligand (Figs. 1*a* and 1*b*). Skeletal muscle is not a recognized opiate-containing tissue and was used as a 'negative control' for the assay; it produced a maximum 10 % level of interference (Fig. 1*a*), which is presumably due to non-specific protein interactions at high protein concentrations. This suggests that the displacement produced by the extracted protein of the other tissues is not simply an artefact and represents specific displacement of the

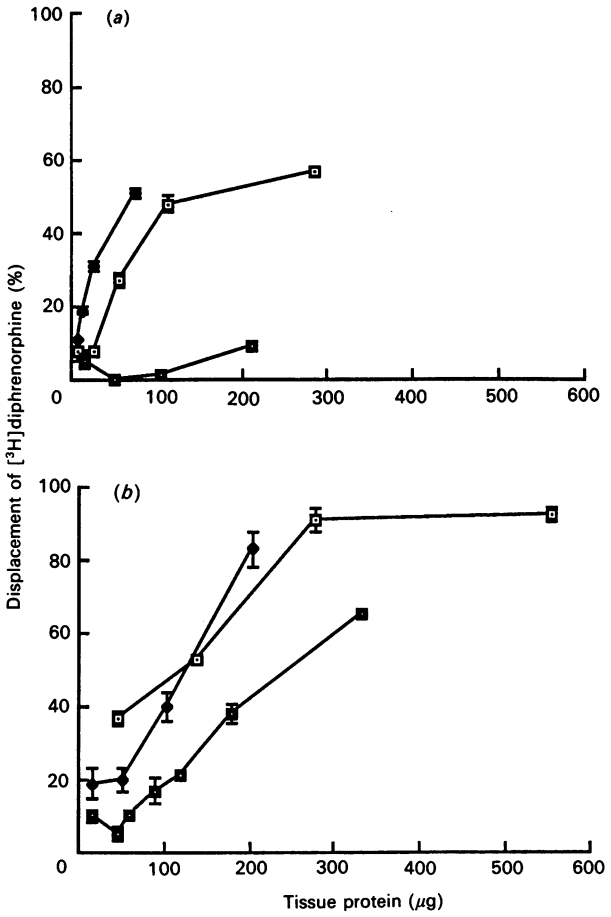

Fig. 1. Displacement of [^{3}H]diprenorphine from brain membranes by tissue extracts

Increasing quantities of extracts of (*a*) brain (⊡), pituitary (◆) and skeletal muscle (■) and (*b*) pancreas (■), liver (⊡) and hepatocytes (◆) were incubated with 1–2 nM-[^{3}H]diprenorphine and brain membranes for 7 h at 4 °C. Non-specific binding was determined by the addition to a parallel series of tubes of 1 μM-naloxone. The level of specific binding was taken as the difference between the radioactivity bound in the presence or absence of naloxone. Separation of membrane-bound and free label was by rapid filtration. Each point represents the mean ± S.E.M. of triplicate determinations from two separate experiments.

Table 1. Displacement (IC_{50}) values and relative potencies of opiates in tissue extracts

IC_{50} values (± S.E.M.) were derived from competitive-displacement curves of [^{3}H]DHM, [^{3}H]DADL and [^{3}H]DYN(1–9) binding to rat brain membranes by extracts of rat brain, pancreas and liver. The assays were performed as described in the legend to Fig. 2 and in the Experimental section. The IC_{50} is defined as the concentration of tissue protein which produces half-maximal inhibition of specific [^{3}H]ligand binding; the most potent activity is arbitrarily defined as 1.0 (values represent the means of six determinations for each tissue).

Tissue	Receptor type...	IC_{50} (μg of protein) μ	δ	κ	Relative potency ($\mu/\delta/\kappa$)
Brain		153±25	107±7	145±17	0.7:1.0:0.7
Pancreas		192±31	243±39	574±92	1.0:0.8:0.3
Liver		263±12	325±60	347±39	1.0:0.8:0.8

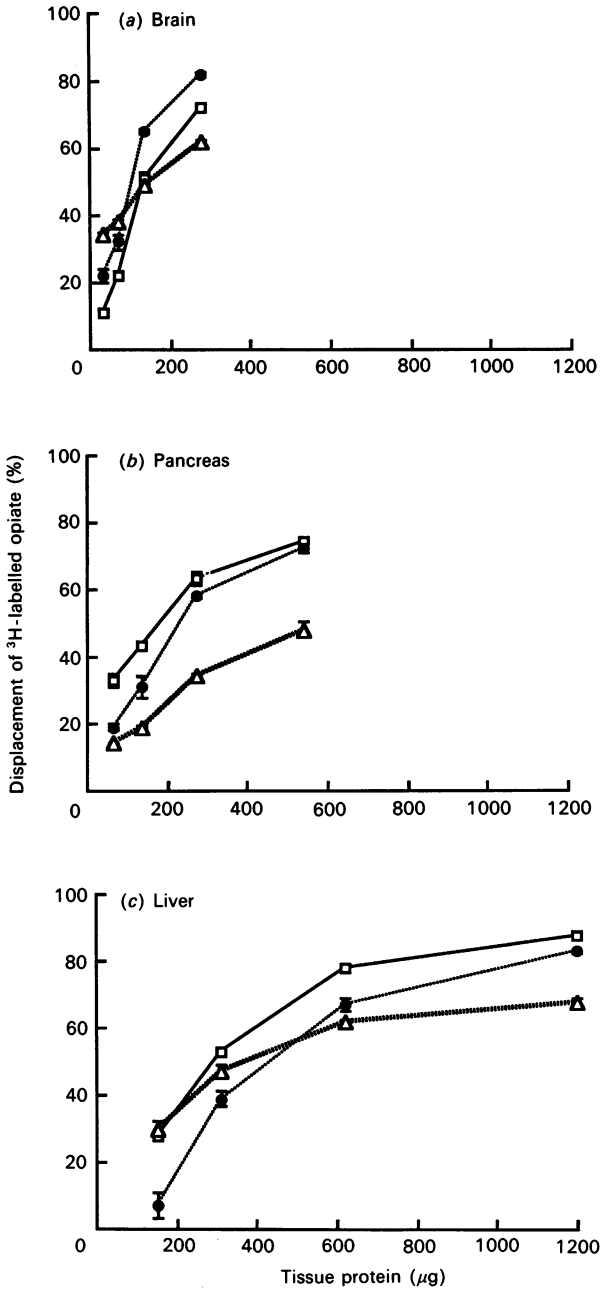

Fig. 2. Displacement of [^{3}H]opiates from rat brain membranes by tissue extracts

Brain membranes were incubated with 1–2 nM-[^{3}H]DHM (□) for 7 h, -[^{3}H]DADL (●) for 4.5 h or -[^{3}H]DYN(1–9) for 1.5 h (△) at 4 °C and with increasing amounts of (*a*) brain, (*b*) pancreas and (*c*) liver extract. Non-specific binding was determined by the addition of 1 μM-DAGO, deltakephalin or dynorphin A-(1–13)-peptide respectively, to a parallel series of tubes. Free and membrane-bound label were separated by rapid filtration. The data were analysed and plotted as described in the Experimental section. The results are means ± S.E.M. of triplicate determinations from two separate experiments.

antagonist from opiate receptors. This confirms the existence of opioid-like peptides in the liver and pancreas (Wajda *et al.*, 1976; Bruni *et al.*, 1979; Houck *et al.*, 1981; Feurle *et al.*, 1982; Smyth & Zakarian, 1982; Stern *et al.*, 1982). The nature of the opioid-receptor-active compounds was investigated further by using labelled selective opiate-receptor agonists. The results in Figs. 2(*a*)–2(*c*) demonstrate the dose-dependent displacement of

Table 2. Quantitative radioreceptor assay of opiates in rat tissues

Brain membranes were incubated with 1–2 nM-[^{3}H]DHM for 7 h, [^{3}H]DADL for 4.5 h [^{3}H]DYN(1–9) for 1.5 h at 4 °C, and with increasing concentrations of DAGO, deltakephalin or dynorphin. Free and membrane-bound label were separated by rapid filtration. The standard curves generated were used to calculate the opioid concentrations in brain liver and pancreas extracts, made and used as described in the Experimental section. Statistical significance of differences from values obtained in brain were determined by unpaired t test: $*P < 0.05$; $**P < 0.02$; $***P < 0.01$. Where μ-, δ- and κ-active compounds are compared in one tissue, the significance of differences in levels is indicated in the Results section. The number of observations was nine except for pancreas μ and κ activities (six) and liver deltakephalin (twelve).

	Receptor-active opiates [pmol/tissue (pmol/g w wt.)]		
Tissue / Receptor type... / Standard used...	μ DAGO	δ Deltakephalin	κ Dynorphin
Brain	72±15 (54±12)	466±134 (355±96)	105±25 (73±17)
Pancreas	30±15 (30±20)	477±79 (429±71)	46±11 (41±10)
Liver	478±174* (47±18)	600±163 (59±15)***	245±42** (24±4)**

specifically bound [^{3}H]DHM, [^{3}H]DADL and [^{3}H]DYN(1–9) from μ, δ and κ opiate receptors respectively, by brain, pancreas and liver extracts. In the case of skeletal-muscle extract, the displacement curves revealed a maximum 10–20% level of interference in the assays with any receptor-specific opiate agonist (results not shown). The data indicate that the opioid peptides present in each tissue are capable of interacting with all three opiate-receptor subtypes. The pattern of μ, δ and κ [^{3}H]ligand displacement by the tissue extracts differed (Table 1). The concentration of extract required to give 50% displacement of the labelled agonists (IC_{50} value) and relative receptor potencies of the extracted opioid peptides were calculated from competitive-displacement curves similar to the one illustrated in Fig. 2. The opioid-like material in brain extract displayed greater selectivity for δ compared with μ or κ opiate receptors, whereas the pancreatic extract exhibited a similar potency at μ and δ opiate receptors, but was two or three times less active at κ opiate receptors (Table 1). On the basis of protein content, there was a definite rank order of μ-, δ- and κ-receptor-active opioid affinity for each receptor subtype dependent on the tissue of origin: brain $\gg$ pancreas > liver (Table 1). This correlates with the result of Wajda *et al.* (1976), who found that liver extract was only 71% as active as that of brain. By comparison of the ability of the extracts to displace the labelled receptor-specific agonists with standard curves produced by incubating the labelled membranes with increasing concentrations of unlabelled DAGO, deltakephalin or dynorphin A-(1–13)-peptide, estimates of the concentrations of μ-, δ- and κ-receptor-specific opioid peptides within the extracts can be obtained (Table 2). The results in Table 2 show that the brain extract contains significantly higher levels of δ- than μ- ($P < 0.01$; $n = 18$; t test) or κ-($P < 0.02$; $n = 18$; t test) receptor-active opioids. Similarly, in pancreas extract, a significantly higher level of δ- than μ- ($P < 0.001$; $n = 15$; t test) or κ- ($P < 0.001$; $n = 15$; t test) receptor-active opioids is found. In liver extracts, the levels of both μ- and δ-receptor-active opioids were similar; however the level of κ-receptor-active opioids was significantly lower than that of the δ-receptor-active opioids ($P < 0.05$; $n = 21$; t test) (Table 2). If one compares the distribution of each type of receptor-active opioid between the three tissues, the levels of μ, δ and κ receptor-active opioids are not significantly different for brain and pancreas (Table 2) and, since the potency profiles between the two tissues differ (Table 1), it can be inferred that different native opioid peptides are present in pancreas and brain extracts. On a weight basis (pmol/g wet wt.), δ- and κ-, but not μ-, receptor-active opiates are significantly lower in liver than in brain; however the total tissue content of μ- and κ-, but not δ-, receptor-active opioids in liver is significantly higher compared with brain and pancreas (Table 2).

Radioimmunoassay

Although the radioreceptor assay gives some indication of the nature of the endogenous opioid peptides present, the chemical identity cannot be confirmed, nor does it allow for cross-reactivity of the endogenous opioid peptides at the different receptor subtypes. Therefore radioimmunoassays were performed to characterize some of the opioid peptides present in extracts of brain, pituitary, pancreas, liver and hepatocytes (Table 3). [Met]enkephalin values obtained for the tissue extracts showed good correlation with estimates reported by other workers: 0.88 pmol of [Met]enkephalin/mg of brain protein (Cuello, 1983); 4320 pmol of [Met]enkephalin/g wet wt. of pituitary (Rossier *et al.*, 1977); 1.62 pmol of [Met]enkephalin/pancreas (Timmers *et al.*, 1986). Most of the [Met]enkephalin is present primarily in the form of high-molecular-mass enkephalins (such as BAM-22P) (Timmers *et al.*, 1986). Very low amounts of MERF immunoreactivity have been reported in extracts of rat pancreas (Tang *et al.*, 1982), but neither MERF nor MERGL were detectable in pancreas in our studies. This contrasts with extracts of rat brain, in which the amounts of MERF and MERGL were 2.2 pmol/brain and 1.2 pmol/brain respectively. Pancreatic [Met]enkephalin levels were similar to, and β-endorphin levels markedly higher than, brain levels (Table 3); this strengthens the notion that a physiological regulatory role is played by such opioid peptides in the pancreas. Good correlation was also found in the case of β-endorphin: 31.7 pmol of β-endorphin/g wet wt. of brain (Cuello, 1983); 77633 pmol of β-endorphin/g wet wt. of pituitary (Rossier *et al.*, 1977). Large discrepancies, however, were observed for β-endorphin measurements in extracts of pancreas; 1.72 fmol/mg of protein (which includes higher-molecular-mass forms) (Timmers *et al.*, 1986); this is equivalent to 0.2 pmol/pancreas compared with 5.8 pmol/pancreas (Vuolteenaho *et al.*, 1980) and 170 pmol/pancreas (Table 3). Differences between these values may be accounted for by the varied methods of tissue extraction used and by the specificity of the antibody for a particular opioid-peptide species. It is evident

Table 3. Radioimmunoassay of β-endorphin and [Met^5]enkephalin in rat tissues

Tissue extracts were prepared and radioimmunoassayed for β-endorphin and [Met^5]enkephalins as described in the Experimental section. Values are the means of triplicate determinations.

	β-Endorphin			[Met^5]Enkephalin		
Tissue	(pmol/ tissue)	(pmol/g wet wt.)	(pmol/ mg of tissue protein)	(pmol/ tissue)	(pmol/g wet wt.)	(pmol/ mg of tissue protein)
Brain	38.5	26.1	0.59	11.9	8.1	0.18
Pituitary	610	59532	311	105	10500	53.6
Pancreas	170	212	—	8.1	10.1	—
Liver	51	6.1	0.13	45.5	5.4	0.11
Hepatocytes	70.5	10.1	0.25	36.3	5.2	0.13

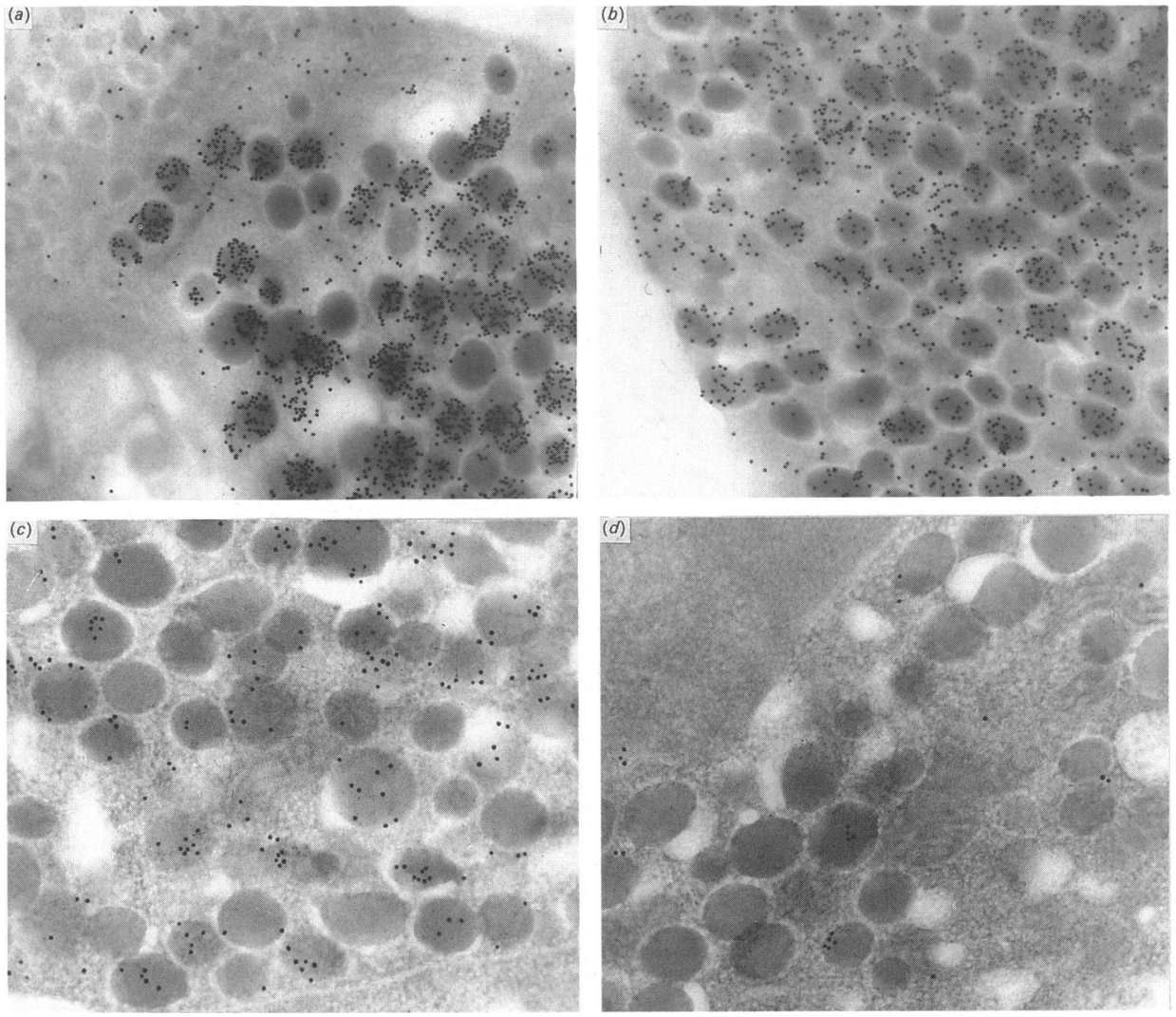

Fig. 3. Immunogold labelling of endogenous opiates in glucagon-containing cells of rat islets

Rat islets were fixed and embedded in LR Gold resin for immunogold labelling (see the Experimental section). Islet sections were incubated with (*a*) glucagon antiserum (1:200), (*b*) anti-dynorphin or (*c*) β-endorphin antiserum (1:80) and (*d*) normal rabbit non-immune serum (1:80). Glucagon and dynorphin were revealed by using Protein A-gold; β-endorphin and normal rabbit serum by using goat anti-rabbit–gold. Magnifications: (*a*) and (*b*) ×40000; (*c*) and (*d*) ×44000.

that both liver and hepatocytes contain substantial amounts of both β-endorphin and [Met^5]enkephalin (Table 3); however, comparatively low levels of MERF were detected in extracts of liver (0.23 pmol/tissue; 0.027 pmol/g wet wt; 0.00058 pmol/mg of tissue protein) and hepatocytes (0.14 pmol/tissue; 0.020 pmol/g wet wt; 0.00050 pmol/mg of tissue protein). No immunoreactive MERGL was detectable in either liver or hepatocytes. The fact that no difference was detected in the opiate activity of both perfused whole liver and isolated hepatocytes, as demonstrated by radioreceptor assay (Table 2) and radioimmunoassay (Table 3), implies that the opioid peptides were not derived from contamination by blood or sympathetic innervation, or non-parenchymal cells, as most of the latter are lost during the preparation of the hepatocytes. This indicates a possible intracellular synthesis and localization of opioids within the parenchymal cells. It is possible that hormonal-type opioids with long half-lives, such as β-endorphin, are taken up from the peripheral circulation and accumulated within this organ until degraded. This is unlikely to be the case with peptides such as [Met^5]enkephalin and [Leu^5]enkephalin, since they possess very short half-lives and are rapidly degraded by the liver (Leach *et al.*, 1985); however, the more metabolically stable pro-enkephalin A-derived opioids (e.g. BAM-22P, peptide E) may be taken up from the peripheral circulation and degraded to the shorter enkephalin residues, primarily [Met^5]enkephalin.

Immunocytochemistry

Immunohistochemical studies were performed on liver and pancreas sections in an attempt to localize such opioid peptides *in situ*. Tissues were fixed with *p*-benzoquinone, sectioned and processed for immunoperoxidase staining. Despite the fact that both β-endorphin and [Met^5]enkephalin were detected in liver by radioimmunoassay, no positive staining for β-endorphin, dynorphin or [Leu^5]enkephalin was detectable in liver sections. In contrast, positive β-endorphin and dynorphin, but not [Leu^5]enkephalin, staining was seen in peripheral cells of rat islets in pancreas sections (results not shown), confirming the reports of other workers (Grube *et al.*, 1978; Watkins *et al.*, 1980; Cetin,

1985; Timmers *et al.*, 1986). The lower concentrations of radioimmunoassayable β-endorphin in liver (20–30-fold less) as compared with pancreas (Table 3) may be below the limit of detection in the immunoperoxidase staining procedure. A similar explanation may be given for the lack of positive dynorphin staining in liver sections, considering the low concentrations (pmol/g wet wt.) of κ-receptor-active opioids in this tissue versus pancreas and brain (Table 2). We attempted a more precise localization of these peptides at the electron-microscopical level by using sections prepared from rat islets of Langerhans. Fig. 3(*a*) shows Protein A-gold labelling of glucagon granules within a single glucagon-containing cell of a rat islet section. Fig. 3(*b*) represents Protein A-gold labelling of glucagon granules with dynorphin-directed antiserum within the same glucagon-containing cell as that illustrated in Fig. 3(*a*). The labelling was directed specifically against the glucagon granules, which infers a selective localization of dynorphin within this islet-cell type. Fig. 3(*c*) shows goat anti-rabbit gold-conjugated anti-β-endorphin labelling of glucagon granules in a rat islet section. Fig. 3(*d*) depicts goat anti-rabbit gold labelling of sections of glucagon cells incubated with normal rabbit serum, which illustrates a very low level of non-specific binding to the sections.

Therefore, the radioreceptor-active dynorphin (Table 2) and radioimmunoassayable β-endorphin (Table 3) reported for pancreatic tissue may arise from dynorphin and β-endorphin in non-insulin-containing islet cells, primarily the glucagon-containing cells.

Opiate-receptor binding sites

There is much experimental work *in vitro* to support a direct opiate-receptor-mediated action of δ-opiate agonists (β-endorphin, enkephalin) (Ipp *et al.*, 1978; Ryder *et al.*, 1980; Curry *et al.*, 1987) and of κ-opiate agonists (Green *et al.*, 1983*b*; Toyota *et al.*, 1985) to stimulate insulin release, at physiological concentrations between 1 and 10 nM, from rat islets of Langerhans. Similarly, several reports exist showing direct effects of opiate agonists on liver function of the rat *in vitro* (Allan *et al.*, 1983; Matsumura *et al.*, 1984). However, there are very few observations documenting the presence of specific opiate receptors in these tissues. In the present study, [^{3}H]DPN, [^{3}H]DHM, [^{3}H]DADL and [^{3}H]DYN(1–8) were used to identify specific opiate-binding sites on rat liver and islet membranes. High specific binding of all three labels was observed in brain membranes at relatively low concentrations of label (4–5 nM) (Table 4). Binding studies using islet membrane preparations demonstrated statistically significant binding of selective δ and κ-[^{3}H]opiates to their respective binding sites. No significant binding of [^{3}H]DHM to μ sites of high affinity (4–5 nM) was measured in crude islet membranes (Table 4). Since collagenase digestion of the pancreas was necessary to obtain islets, the deleterious effects of the enzyme on opiate binding was tested in rat brain membranes (Table 5). In general, collagenase reduces non-specific binding; for [^{3}H]DHM and [^{3}H]DYN(1–8) these are significant (Table 5). The level of specific binding of [^{3}H]DHM to μ receptors was significantly raised ($P < 0.02$) in the collagenase-treated preparations, which was primarily due to a reduction in the level of non-specific binding (Table 5). These findings confirm that no deleterious effects of collagenase on opiate-receptor binding is produced. δ, but not μ, opiate-binding sites were shown previously to be present in [^{3}H]agonist-uptake studies in intact rat islets of Langerhans (Verspohl *et al.*, 1986). The present study confirms the existence of δ sites and, for the first time, demonstrates the presence of κ opiate-binding sites in islets of Langerhans. These observations are consistent with published findings on biological efficacy of different opiate agonists in increasing insulin release (Ipp *et al.*, 1978; Green *et al.*, 1980; Kanter *et al.*, 1980). Experiments similar to those reported above for brain and islet membranes were carried out on crude liver membranes as well as purified liver plasma membranes in an attempt to identify specific opiate-binding sites. No specific binding could be detected with any of the labelled receptor-selective agonists. Increasing the amount of liver homogenate protein added per assay tube from 0.5 mg to a maximum of 7.0 mg per tube, in order to raise the receptor concentration, had no effect on the binding of the δ selective agonist, [^{3}H]DADL (2 nM). This label was selected in preference to others, since δ agonists such as [Leu^5]enkephalin have been shown to exert direct effects on hepatocyte glucose production (Leach *et al.*, 1985). Saturation binding studies performed at 4 °C with [^{3}H]DPN over the concentration range 1–20 nM and using 200–300 μg of purified liver plasma membrane per assay tube failed to demonstrate either high- or low-affinity opiate-binding sites (results not shown). Evidence for the existence of non-opiate receptors in rat hepatic membranes which are specific for β-endorphin has been provided by Dave *et al.* (1985). We did not attempt any studies using β-endorphin as displacer of bound [^{3}H]DPN to rat liver membranes. However, we did find that high

Table 4. Selective opiate-binding sites in brain and islets of Langerhans

Brain and islet tissue (200 μg/tube) were incubated at room temperature for 3 h with [^{3}H]DHM (4–6 nM), and [^{3}H]DADL (4–5 nM), and for 20 min with [^{3}H]DYN(1–8) (5–6 nM). Non-specific binding was determined with excess unlabelled DAGO, deltakephalin and dynorphin respectively. Separation of bound and free ligand was by microcentrifugation. Further details are given in the Experimental section. The results represent the means for two or three experiments (six determinations). (S.E.M. values are < 1 % of the means and are not shown). The data was analysed statistically by comparing the total radioactivity bound and non-specifically bound; * and ** represents significant specific binding of ^{3}H-labelled ligand: * $P < 0.05$; ** $P < 0.01$ (by Student's unpaired *t* test).

Site	Opiate binding (fmol/mg of protein)	
	Brain	Islets
[^{3}H]DHM-specific	93.7**	5.6
[^{3}H]DADL-specific	139.3**	16.5**
[^{3}H]DYN(1–8)-specific	92.4**	39.1*

Table 5. Effect of collagenase digestion on [^{3}H]opiate binding

Brain membranes (1 mg/ml) were incubated at room temperature for 3 h with [^{3}H]DHM (2 nM) and [^{3}H]DADL (1 nM) and for 20 min with [^{3}H]DYN(1–8) (1 nM). Non-specific binding was determined with excess unlabelled DAGO, deltakephalin and dynorphin A-(1–13)-peptide respectively. Separation of bound and free ligand was by microcentrifugation. Values (mean ± S.E.M.) significantly different from control: * $P < 0.02$; ** $P < 0.01$ (by Student's unpaired *t* test) (three determinations).

Ligand	Treatment	Binding (fmol/mg of protein)		
		Total bound	Non-specific	Specific
[^{3}]DHM	Control	12.7±1.2	6.7±0.3	6.0±0.6
	Collagenase	13.6±0.4	4.9±0.2**	8.7±0.2*
[^{3}H]DADL	Control	74.7±8.1	40.2±2.6	34.5±4.3
	Collagenase	76.4±1.3	29.4±5.1	47.0±2.6
[^{3}H]DYN(1–8)	Control	35.7±2.3	19.6±1.1	16.0±1.3
	Collagenase	29.8±7.0	14.1±0.6	15.6±3.5

concentrations of [^{3}H]DPN (73–74 nM/tube) could be displaced, as much as 20–30% of total binding, by 1 μM-angiotensin II and 1 μM-vasopressin, as well as by 1 μM-DAGO, deltakephalin and dynorphin A-(1–13)-peptide; this was considered as non-specific displacement of membrane-bound radioligand, but could represent some other receptor type, not necessarily opiate in nature. Many of the effects of opioid peptides on hepatic carbohydrate metabolism are not inhibited by naloxone, a non-specific opiate-receptor antagonist (Allan *et al.*, 1983; Leach *et al.*, 1985). Hepatic carbohydrate metabolism is influenced by the hormonal actions of adrenaline, vasopressin, glucagon and angiotensin II acting at their respective receptor sites. [Leu^5]enkephalin and [Met^5]enkephalin were capable of displacing specifically bound [^{125}I]angiotensin II from liver membranes (Titheradge & Hothi, 1987; Hothi *et al.*, 1989), and may produce their biological actions (such as increased glucose output) by a cross-reaction at these sites (Allan *et al.*, 1983; Leach *et al.*, 1985; Hothi *et al.*, 1989).

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