Evidence that the agonist action of dynorphin A(1–8) in the guinea-pig myenteric-plexus may be mediated partly through conversion to [Leu⁵]enkephalin

¹Diane M. Dixon & ²J.R. Traynor

Department of Chemistry, University of Technology, Loughborough, Leics., LE11 3TU

- 1 The agonist action of the opioid peptide dynorphin A(1–8) on the myenteric plexus-longitudinal muscle of the guinea-pig ileum has been characterized.
- 2 The endogenous opioid peptide dynorphin A(1–8) was rapidly degraded by slices of myenteric plexus-longitudinal muscle of the guinea-pig ileum.
- 3 A product of the degradation was the δ -receptor preferring [Leu⁵]enkephalin. Levels of [Leu⁵]enkephalin were markedly increased in the presence of the peptidase inhibitors bestatin, thiorphan and captopril.
- 4 In the myenteric plexus dynorphin A(1–8) acted as a κ -receptor agonist. In the presence of bestatin, thiorphan and captopril a μ -receptor agonist effect was observed. This μ -agonist action was lost in the presence of N-[1-(RS)-carboxy-2-phenylethyl]Ala-Ala-Phe-p-aminobenzoate, an inhibitor of the endopeptidase enzyme EC 3.4.24.15.
- 5 The results suggest that formation of [Leu⁵]enkephalin from dynorphin A(1–8) may be an important conversion process. The enzyme responsible may be the Zn²⁺-metalloendopeptidase, EC 3.4.24.15.

Introduction

The dynorphin family of opioid peptides are considered to be endogenous ligands for the κ -opioid receptor. The larger dynorphin peptides, namely dynorphin A (dynorphin A(1–17)) and dynorphin A(1–13) are potent selective κ -receptor agonists (Chavkin *et al.*, 1982; James *et al.*, 1984). However the shorter peptides, dynorphin A(1–8) and A(1–9), are less potent and less selective (Corbett *et al.*, 1982). In addition, these shorter sequences are highly susceptible to enzymic degradation in homogenates of the central nervous system (CNS) (Gillan *et al.*, 1985; Dixon & Traynor, 1987). Consequently, pharmacological studies of these smaller dynorphin fragments are performed in the presence of a cocktail of peptidase inhibitors to protect the C- and N-termini and the Gly³-Phe⁴ bond from hydrolysis (Corbett *et al.*, 1982; McKnight *et al.*, 1983). However, even in the presence of such a cocktail, [Leu⁵]enkephalin may be produced (Miller *et al.*, 1985; Dixon & Traynor, 1987) and this pentapeptide may contribute to the observed pharmacological actions of the dynorphin fragments (Miller *et al.*, 1985). Indeed the peptidase inhibitors may stabilize [Leu⁵]enkephalin and therefore favour formation of this pentapeptide (Dixon & Traynor, 1987). Cleavage of the Leu⁵-Arg⁶ bond of dynorphin A(1–8), which gives rise to [Leu⁵]enkephalin, can be prevented by the EC 3.4.24.15 inhibitor N-[1-(RS)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB (Chu & Orłowski, 1984; Dixon & Traynor, 1990).

The formation of [Leu⁵]enkephalin from the smaller dynorphins would have important consequences if it occurred in physiological situations. As a preliminary to such investigations, we describe the formation of [Leu⁵]enkephalin from exogenously added dynorphin A(1–8) in the myenteric plexus of the guinea-pig ileum, and its role in the observed agonist activity.

Methods

Male Dunkin-Hartley guinea-pigs (400–500 g) (David Hall, Burton-on-Trent) were killed by cervical dislocation. Seg-

ments of ileum were removed and placed in Krebs solution containing (mM): NaCl 118, KCl 4.7, CaCl₂ · 2H₂O 2.6, KH₂PO₄ 1.2, MgSO₄ · 7H₂O 1.2, NaHCO₃ 25 and glucose 11.

Bioassays

The myenteric plexus-longitudinal muscle preparation of the guinea-pig small intestine was set up for field stimulation as previously described (Traynor *et al.*, 1987). The potencies of agonists were obtained from cumulative dose-response curves. The equilibrium dissociation constants (K_e , nM) of the antagonists were determined by the method of Arunlakshana & Schild (1959) or by the 'single-dose' method (Kosterlitz & Watt, 1968). Antagonists were allowed to equilibrate for 20 min, or 45 min for norbinaltorphimine, before repeating the addition of agonist.

Pre-incubation of myenteric plexus preparations with peptidase inhibitors

Myenteric plexus preparations were incubated in Krebs solution as above with the peptidase inhibitors bestatin (30 μ M), captopril (10 μ M), thiorphan (0.3 μ M) (McKnight *et al.*, 1983) and, where stated, N-[1-(RS)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB (30 μ M) (Chu & Orłowski, 1984) for 30 min. Following this treatment potencies of agonists were determined without washout of the peptidase inhibitors.

Pre-incubation of myenteric plexus preparations with β -funaltrexamine

To block μ -opioid receptors irreversibly, preparations of myenteric plexus were incubated with β -funaltrexamine (100 nM) for 60 min and then washed with drug-free Krebs solution for 60 min. During washing the bath fluid was changed by overflow every 5 min. Agonist potencies were determined before and after β -funaltrexamine treatment.

Metabolism studies

Preparations of myenteric plexus-longitudinal muscle from the guinea-pig ileum were sliced (0.5 mm) on a McIlwain tissue chopper. The slices were washed three times in Krebs-solution

¹ Present address: Department of Physiology and Pharmacology, Medical School, Queen's Medical Centre, Nottingham.

² Author for correspondence.

containing HEPES buffer (pH 7.4, 25 mM) (Krebs-HEPES) and then suspended in Krebs-HEPES at a tissue concentration of 200 mg ml⁻¹. Aliquots of tissue slices (10 mg) were incubated in Krebs-HEPES solution (1 ml) in the presence or absence of peptidase inhibitors for 30 min at 37°C, prior to the addition of [³H]-dynorphin A(1-8) at a final concentration of 12 nM. At timed intervals subsequent to this the reaction was terminated by the addition of phosphoric acid (50 mM final concentration). Aliquots (500 µl) were taken and stored at -20°C prior to analysis.

To aliquots of the incubation mixtures were added 20 µl of a solution containing the marker peptides Tyr, Tyr-Gly, Tyr-Gly-Gly, Tyr-Gly-Gly-Phe, [Leu⁵]enkephalin, [Leu⁵]enkephalyl-Arg⁶, [Leu⁵]enkephalyl-Arg⁶-Arg⁷, and dynorphin A(1-8) each at a concentration of 100 µg ml⁻¹. The samples were applied to an Altex Ultrasphere ODS column (250 mm × 4.6 mm, particle size 5 µm) and eluted using a solvent gradient between solutions A (26 mM trifluoroacetic acid with triethylamine to pH 3.0) and B (49% acetonitrile in 13 mM trifluoroacetic acid with triethylamine to pH 3.0) as follows: 15–30%B in 15 min: 30–60%B in 30 min: 65–70%B in 5 min, at a flow rate of 1.0 ml min⁻¹. Peaks were detected at 280 nm and fractions (0.5 ml) collected from the column. The gradient system used did not readily separate the Tyr, Tyr-Gly, and Tyr-Gly-Gly fractions which were collected as a single 'N-terminal fraction'. Radioactivity in each fraction was determined after addition of 4 ml of scintillation cocktail (Ecoscint, National Diagnostics). Recovered radioactivity represented 94.1 ± 8.0% (n = 25) of the added activity.

Peptides and drugs

[³H]-dynorphin A(1-8) (27.6 Ci mmol⁻¹) was obtained from New England Nuclear. Peptides were from Cambridge Research Biochemicals with the exception of Tyr, Tyr-Gly and Tyr-Gly-Gly which were from Sigma. Peptidase inhibitors were obtained from the following sources: bestatin (Cambridge Research Biochemicals), captopril (Squibb), thiorphan (Sandoz) and N-[1-(RS)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB (where pAB = *p*-aminobenzoate, Professor M. Orłowski, Mount Sinai, New York, U.S.A.). The following drugs were used: [D-Ala², MePhe⁴, Gly-ol⁵]enkephalin (DAMGO, Sigma); *trans*, 3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]-benzeneacetamide (U-50488H) and 5α,7α,8β-(–)-N-methyl-N-(7-(1-pyrrolidinyl)-1-oxaspiro[4,5]-dec-8-yl)benzeneacetamide (U-69,593, Upjohn); β-funaltrexamine, (β-FNA, Research Biochemicals Inc.); naloxone hydrochloride (Endo Laboratories), 16-methylcyprenorphine (M8008, Reckitt and Colman); norbinaltorphimine (Glaxo). All other reagents were of analytical, or high performance liquid chromatography (h.p.l.c.) grade.

Results

Metabolism of [³H]-dynorphin A(1-8) by the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum

Incubation of [³H]-dynorphin A(1-8) (12 nM) with slices of myenteric plexus-longitudinal muscle at 37°C resulted in a rapid degradation of the tritiated octapeptide as determined by h.p.l.c. separation of all the possible tritiated products (Figure 1a). The half-life of [³H]-dynorphin under these conditions was just 2.30 ± 0.46 min. The major products of the breakdown co-eluted with the Tyr, Tyr-Gly and Tyr-Gly-Gly markers. Radioactivity was also recovered in the fraction co-eluting with the [Leu⁵]enkephalin marker. This product reached peak levels of 8.2 ± 1.2% of recovered activity after

Figure 1 Metabolism of [³H]-dynorphin A(1-8) (12 nM) by slices of myenteric plexus-longitudinal muscle of the guinea-pig ileum in the absence (a) or presence (b) of the peptidase inhibitors bestatin (10 µM), captopril (10 µM) and thiorphan (0.3 µM). [³H]-dynorphin A(1-8) (●); [³H]-[Leu⁵]enkephalin (■); [³H]-N-terminal fraction (▲). Points represent means of three determinations; vertical bars show s.e.means.

10 min, but then declined. Other products of the metabolism eluting with the Tyr-Gly-Gly-Phe, [Leu⁵]enkephalyl-Arg⁶ and [Leu⁵]enkephalyl-Arg⁶-Arg⁷ markers accounted for less than 5% of the recovered radioactivity.

In the presence of the peptidase inhibitors bestatin (30 µM), captopril (10 µM) and thiorphan (0.3 µM) (McKnight *et al.*, 1983) the half-life of [³H]-dynorphin A(1-8) in the presence of slices of myenteric plexus-longitudinal muscle increased to 7.0 ± 1.1 min. This was the result of a marked decline in the radioactivity attributed to the 'N-terminal' (Tyr, Tyr-Gly, Tyr-Gly-Gly) products. However, the level of radioactivity identified as [³H]-[Leu⁵]enkephalin was much increased and represented the major product of [³H]-dynorphin A(1-8) metabolism accounting for 41.7 ± 1.0% of the recovered activity after 20 min (Figure 1b). There was no alteration in the levels of [³H]-Tyr-Gly-Gly-Phe, [³H]-[Leu⁵]enkephalyl-Arg⁶ and [³H]-[Leu⁵]enkephalyl-Arg⁶-Arg⁷ recovered.

Agonist action of dynorphin A(1-8) in the myenteric plexus longitudinal muscle preparation of the guinea-pig ileum

Dynorphin A(1-8) in the absence of peptidase inhibitors was able to depress the electrically-induced contractions of the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum, a tissue containing both µ- and κ-opioid receptors (Chavkin & Goldstein, 1981). The peptide acted as a full agonist in this tissue (Table 1). The action of dynorphin A(1-8) was antagonized by naloxone affording an equilibrium dissociation constant (K_e) for the antagonist of 14 nM. This value is in agreement with the K_e for naloxone against the selective κ-agonists U-50488H, U-69,593 and dynorphin A rather than against the µ-selective agonist DAMGO or [Leu⁵]enkephalin (Table 1).

Table 1 The agonist actions of dynorphin A(1–8) and other opioids on the electrically induced contractions of the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum and their antagonism by naloxone

Opioid	Peptidase inhibition*	Agonist activity (IC ₅₀ , nM)	Antagonism by naloxone (K _e , nM)	Antagonism by naloxone (Slope)
Dynorphin A(1–8)	No	27.4 ± 3.7	14.0 ± 2.4	0.92 ± 0.03 (19)
Dynorphin A(1–8)	Yes	1.80 ± 0.34	3.01 ± 0.79	0.86 ± 0.08 (9)
Dynorphin A(1–8) with E.C. 3.4.24.15 inhibitor†	Yes	1.89 ± 0.72	14.3 ± 2.1	single dose (3)
DAMGO	No	15.2 ± 0.14	2.09 ± 0.54	0.92 ± 0.04 (3)
DAMGO	Yes	21.3 ± 7.4	3.07 ± 0.82	1.00 ± 0.03 (3)
DAMGO with E.C. 3.4.24.15 inhibitor†	Yes	16.7 ± 0.90	3.00 ± 0.57	single dose (3)
[Leu ⁵]enkephalin	Yes	36.8 ± 7.0	1.63 ± 0.34	0.83 ± 0.03 (3)
U-50488H	No	1.52 ± 0.14	19.6 ± 1.4	single dose (6)
U-69,593	No	2.80 ± 0.47	31.4 ± 9.7	0.98 ± 0.08 (4)
Dynorphin A	No	0.32 ± 0.02	33.6 ± 1.02	1.07 ± 0.03 (4)

The values are the means ± s.e.mean; the number of observations is given in parentheses. * Bestatin (30 μM), captopril (10 μM) and thiorphan (0.3 μM).

† N-[1-(RS)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB (30 μM). Individual preparations of myenteric plexus were pre-incubated with peptidase inhibitors for 30 min prior to determination of agonist potency and degree of antagonism by naloxone.

DAMGO: [D-Ala², MePhe⁴, Gly-ol⁵]enkephalin.

In the presence of the peptidase inhibitors bestatin (30 μM), captopril (10 μM) and thiorphan (0.3 μM) (McKnight *et al.*, 1983), the agonist potency of dynorphin A(1–8) was increased 15 fold. The K_e for naloxone was also shifted to give a value similar to that obtained against DAMGO in the presence and absence of peptidase inhibitors, and [Leu⁵]enkephalin (Table 1). When the EC 3.4.24.15 inhibitor N-[1-(RS)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB (30 μM) (Chu & Orlowski,

1984) was added to the bath fluid, in addition to the standard inhibitors, the potency of dynorphin A(1–8) as an agonist did not change. However the K_e for naloxone against dynorphin A(1–8) reverted back to a value seen in the absence of any peptidase inhibitors (Table 1). N-[1-(RS)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB (30 μM) had no effect on the IC₅₀ for DAMGO or on the naloxone K_e determined against DAMGO (Table 1).

Table 2 The effectiveness of 16-methylcyprenorphine (M8008) and norbinaltorphimine in antagonizing the agonist actions of opioids in the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum

Compound	16-Methylcyprenorphine		Norbinaltorphimine	
	K _e (nM)	Slope	K _e (nM)	Slope
DAMGO	3.50 ± 1.21	0.91 ± 0.05 (4)	13.0 ± 1.91	1.04 ± 0.08 (5)
U-69,593	85.8 ± 14.4	0.96 ± 0.11 (4)	0.07 ± 0.01	1.07 ± 0.03 (3)
Dynorphin A(1–8)	76.9 ± 11.1	0.91 ± 0.03 (4)	0.17 ± 0.04	0.98 ± 0.08 (3)
Dynorphin A(1–8) with peptidase inhibition*	31.4 ± 8.81	0.79 ± 0.01** (4)	0.62 ± 0.07	1.07 ± 0.03 (7)

The values are means ± s.e.mean; the number of observations is given in parentheses.

* Bestatin (30 μM), thiorphan (0.3 μM), captopril (10 μM).

** Significantly less than unity ($P < 0.05$) (Mann-Whitney).

DAMGO: [D-Ala², MePhe⁴, Gly-ol⁵]enkephalin.

Table 3 The effects of preincubation with β-funaltrexamine on the agonist potencies of opioids in the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum

Opioid	Agonist potency (IC ₅₀ , nM)		
	Before β-FNA	After β-FNA	Dose-ratio
Dynorphin A(1–8)	18.6 ± 5.6	11.6 ± 2.5	0.6 ± 0.1 (3)
Dynorphin A(1–8) in presence of peptidase inhibition*	1.2 ± 0.35	1.57 ± 0.27	1.6 ± 0.4 (6)
DAMGO	12.2 ± 1.8	263 ± 48.8	26.1 ± 7.5 (12)
[Leu ⁵]enkephalin in presence of peptidase inhibition*	33.1	552	15.9 (2)
U-50488H	1.73 ± 0.56	2.1 ± 0.2	1.1 ± 0.5 (4)

The values are means ± s.e.mean; the number of observations is given in parentheses. Individual preparations of myenteric plexus were pre-incubated with 100 nM β-funaltrexamine (β-FNA) for 60 min, then washed for 60 min with drug-free Krebs solution. Dose-ratio is the ratio of IC₅₀ after preincubation with β-FNA to IC₅₀ before treatment.

*Bestatin (30 μM), thiorphan (0.3 μM), captopril (10 μM). DAMGO: [D-Ala², MePhe⁴, Gly-ol⁵]enkephalin.

The antagonist properties of the more selective antagonists 16-methylcyprenorphine (M8008), which is selective for μ - and δ - over κ -opioid receptors (Smith, 1987), and nornalbinaltorphimine which is selective for κ - over μ - and δ -opioid receptors (Portoghese *et al.*, 1987), are given in Table 2. The K_e value for M8008 against dynorphin A(1-8) agrees with the K_e determined against the selective κ -agonist U-69,593 but differs from that obtained against the μ -receptor ligand DAMGO. In the presence of bestatin (30 μ M), captopril (10 μ M) and thiorphan (0.3 μ M) the K_e shifted to a value midway between that determined against U-69,593 and the selective μ -agonist DAMGO (Table 2) and the slope of the Schild plot shifted to a value less than 1.0. The κ -selective antagonist nornalbinaltorphimine showed a similar pattern of activity (Table 2).

Effects of pre-incubation with β -funaltrexamine on the agonist activity of dynorphin A(1-8) in the guinea-pig myenteric plexus

Blockade of μ -receptors by pretreatment with β -FNA did not alter the agonist potency of dynorphin A(1-8) in the myenteric plexus, either in the absence or presence of the peptidase inhibitors bestatin (30 μ M), captopril (10 μ M) and thiorphan (0.3 μ M). The agonist potency of U-69,593 was similarly unchanged. However, the dose-response curves for DAMGO and [Leu⁵]enkephalin were markedly shifted to the right (Table 3).

Discussion

The results demonstrate that the endogenous opioid peptide dynorphin A(1-8), a κ -opioid receptor agonist (Corbett *et al.*, 1982), is rapidly metabolized by slices of myenteric plexus-longitudinal muscle preparation from the guinea-pig ileum. One product of this metabolism is the smaller δ -receptor preferring [Leu⁵]enkephalin, which has little affinity for κ -opioid receptors (Gillan & Kosterlitz, 1982). This is similar to findings in CNS tissue (Griffiths *et al.*, 1983; Gillan *et al.*, 1985; Dixon & Traynor, 1987; 1990) and mouse vas deferens (Miller *et al.*, 1985). Since the process in myenteric plexus-longitudinal muscle occurs in well-washed tissue slices it is unlikely to be due to non-specific hydrolases such as might be released by damaged cells.

The rate of breakdown of dynorphin A(1-8) is retarded in the presence of the peptidase inhibitors captopril, bestatin and thiorphan (McKnight *et al.*, 1983) in accordance with the findings of Corbett *et al.* (1982). These inhibitors protect the C and N termini and the Gly³-Phe⁴ bond, respectively, of the larger peptide. However the inhibitors do not seem to prevent cleavage of dynorphin A(1-8) at the Leu⁵-Arg⁶ bond. Breakage of this bond leads to the production of [Leu⁵]enkephalin, which in the presence of the peptidase inhibitors becomes the major tyrosine containing product. Since there is no evidence that significant amounts of [Leu⁵]enkephalyl-Arg⁶-Arg⁷ or [Leu⁵]enkephalyl-Arg⁶ are produced under the assay conditions, the findings support our earlier suggestion (Dixon & Traynor, 1987) that a single cleavage step is involved, rather than sequential breakdown from the C-terminus. It also is clear that the peptidase inhibitors stabilize the [Leu⁵]enkephalin formed from further metabolism thus leading to an accumulation of this pentapeptide.

The [Leu⁵]enkephalin formed from exogenously added dynorphin A(1-8) appears to play a role in the observed agonist action of dynorphin A(1-8) in the myenteric plexus. The action of dynorphin A(1-8) in this tissue is antagonized by naloxone. The affinity of naloxone for the receptors at which dynorphin A(1-8) is acting is low and similar to the naloxone affinity (K_e) at κ -opioid receptors as measured

against the selective synthetic κ -receptor agonists U-69,593 and U-50488H and the endogenous stable peptide dynorphin A. Thus, although dynorphin A(1-8) is a relatively non-selective ligand (Corbett *et al.*, 1982) in the myenteric plexus, it appears to have a preference for the κ -receptor. On the other hand this profile is changed markedly when the experiments are repeated in the presence of the peptidase inhibitors bestatin, thiorphan and captopril. Under such conditions the potency of dynorphin A(1-8) is greatly increased. Moreover the effectiveness of naloxone in antagonizing the effect is also increased, affording a K_e value similar to that obtained when using the ligands DAMGO, in the presence or absence of the inhibitors, and [Leu⁵]enkephalin which act *via* the μ -opioid receptor. It may be hypothesized that in the presence of the peptidase inhibitors the increased levels and stability of [Leu⁵]enkephalin formed from dynorphin A(1-8) becomes the major determinant of specificity. After addition of the EC 3.4.24.15 enzyme inhibitor N-[1-(RS)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB (Chu & Orłowski, 1984) to the usual cocktail of inhibitors, the potency of dynorphin A(1-8) in the myenteric plexus is not further improved. However the effectiveness of naloxone in antagonizing dynorphin A(1-8) under these conditions is reduced, affording a value for K_e compatible with an interaction via κ -opioid receptors, while the observed parameters with the μ -receptor ligand DAMGO are not altered. This suggests that the EC 3.4.24.15 enzyme inhibitor is stabilizing the dynorphin A(1-8) and preventing formation of [Leu⁵]enkephalin. This idea is supported by metabolism studies which demonstrate that N-[1-(RS)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB is able to protect dynorphin A(1-8) from cleavage at the Leu⁵-Arg⁶ bond. Thus in slices of rat CNS tissue, in the presence of captopril, bestatin and thiorphan, N-[1-(RS)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB (30 μ M) stabilizes dynorphin A(1-8) such that after a 10 min incubation at 37°C, a 70% recovery of the peptide is obtained (Dixon & Traynor, 1990). In the absence of this latter inhibitor less than 10% of the intact peptide is recovered.

It seems clear from the above discussion that the apparent non-selectivity of dynorphin A(1-8) may be attributed, at least partly, to metabolism to the μ/δ -preferring [Leu⁵]enkephalin. Further studies however suggest the action of exogenous dynorphin A(1-8) in the presence of the peptidase inhibitors bestatin, thiorphan and captopril cannot be wholly attributed to an action of formed [Leu⁵]enkephalin at μ -receptors. Thus, results with the selective κ -antagonist nornalbinaltorphimine and the μ/δ -receptor antagonist 16-methylcyprenorphine suggest a mixed action of dynorphin A(1-8) at μ - and κ -opioid receptors. Indeed the slope of the Schild plot for M8008 was less than 1.0. This could have been due to the inherent non-selectivity of the dynorphin A(1-8) or to an action of unmetabolised dynorphin A(1-8) at κ -receptors and formed [Leu⁵]enkephalin at μ -receptors. Thus, after alkylation of μ -receptors with the irreversible antagonist β -funaltrexamine (Corbett *et al.*, 1985), the agonist potency of exogenous dynorphin A(1-8) was not altered, as would have been expected if it had an action at κ -opioid receptors. This suggests that with a large reduction in the number of μ -opioid receptors as shown by a shift in the dose-response curves for DAMGO and [Leu⁵]enkephalin, the observed agonist action of dynorphin A(1-8) was governed by the unmetabolised dynorphin A(1-8) activating κ -opioid receptors, rather than formed [Leu⁵]enkephalin acting at μ -receptors.

A further problem with interpretation of the results is the time course of metabolism of dynorphin A(1-8) to [Leu⁵]enkephalin as compared with the much more rapid onset of agonist action in the myenteric plexus. This may be explained if it is considered that the important [Leu⁵]enkephalin concentration is that in the immediate vicinity of the opioid receptors, rather than the overall concentration in the tissue bath. This would indicate that the enzyme is close to the receptors and therefore affords a high local [Leu⁵]enkephalin concentration.

Several studies have suggested that in certain areas of the CNS [Leu⁵]enkephalin may be formed from prodynorphin, rather than from proenkephalin (Zamir *et al.*, 1983; 1984; Taquet *et al.*, 1985). The present study suggests an extra dimension in that the released dynorphin A (1–8) may be converted to [Leu⁵]enkephalin outside the cell. Whether this is

an important process in controlling the physiological role of dynorphin A(1–8) will have to await further study.

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