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N,N-diallyl-tyrosyl substitution confers antagonist properties on the κ-selective opioid peptide [D-Pro^{10}]dynorphin A(1–11)

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1 In the search for κ-opioid antagonists, we have designed two N,N-diallyl substituted analogues of the κ-selective peptide [D-Pro^{10}]dynorphin A (1–11)(DPDYN). In this study, we have examined (i) the binding properties of N,N-diallyl-DPDYN (analogue 1) and N,N-diallyl-[Aib^{2,3}]DPDYN (analogue 2) at the three main types (μ, δ, κ) of opioid binding sites, (ii) their binding sensitivity to Na^+ ions (120 mM NaCl) and guanine nucleotide (50 μM Gpp(NH)p) at μ- and κ-binding sites and (iii) their biological activity in two pharmacological bioassays specific for μ- and κ-(guinea-pig ileum) and κ-(rabbit vas deferens) opioid receptors.

2 Steric hindrance resulting from incorporation of two bulky allyl groups at the tyrosyl nitrogen atom greatly altered the binding properties of DPDYN. A dramatic fall in apparent affinity for the three types (μ, δ, κ) of site as well as selectivity for κ-sites was observed for the two N,N-diallyl-substituted peptide analogues.

3 At κ-sites of guinea-pig cerebellum and μ-sites of rabbit cerebellum, N,N-diallyl-substitution led to a complete loss of binding sensitivity to the inhibitory effect of 120 mM NaCl + 50 μM Gpp(NH)p compared to the high sensitivity of DPDYN. This may therefore suggest that the N,N-diallyl-DPDYN analogues are endowed with opioid antagonist properties.

4 No agonist activity of the analogues was observed in guinea-pig myenteric plexus and rabbit vas deferens organ preparations. In contrast, both of the diallyl-substituted peptides displayed similar antagonist properties against the κ-agonist DPDYN in both preparations. In the guinea-pig ileum, the affinities of the antagonist peptides against the μ-agonist Tyr-D-Ala-Gly-MePhe-$NH(CH_2)_2OH$(DAGOL) were approximately half that observed against DPDYN.

5 These results show that N,N-diallyl-tyrosyl substitution leads to analogues of DPDYN which act *in vitro* as pure opioid antagonists and exhibit a reasonable affinity at, but a weak selectivity for, the κ-opioid receptors.

Introduction

Selective antagonists for a single receptor type are useful tools for the study of pharmacological and physiological roles played by endogenous (or exogenous) ligands and their receptors. In the case of the opioid system, however, most of the opioid antagonists currently available lack selectivity towards μ-, δ- or κ- (particularly μ vs κ) receptor types. Dynorphin A (dyn A) has been demonstrated to be the putative endogenous ligand of the κ-opioid receptor type (Chavkin *et al.*, 1982) and one can assume that κ-selective antagonist peptides may be obtained after structural modifications in the sequence of dyn A. This idea recently emerged from the fact that, in the case of many biological peptide (hormone)-receptor systems, antagonists are generally analogues derived from the endogenous ligand itself (for reviews, see Manning & Sawyer, 1984; Regoli, 1985). In the case of the opioid receptors, [Ala^2, Trp^4]dyn A (1–13) has been claimed to be a κ-selective opioid antagonist (Lemaire & Turcotte, 1986) and we have shown that D-Trp substitution at positions 2, 4, 5 and 8 in the sequence of [D-Pro^{10}]-

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dyn A (1–11)(DPDYN), a κ-selective analogue of dyn A (Gairin *et al.*, 1984), led to analogues with opioid antagonist properties (Gairin *et al.*, 1986). An alternative approach in the search for opioid antagonist peptides may be to design peptides in which the N-terminal tyrosyl residue possesses an N-allyl substituent, by analogy with that of the nitrogen atom of the piperidine ring of the most powerful alkaloid opiate antagonists. In this way, a single substitution with the allyl moiety has been shown to induce only mixed agonist-antagonist properties to [D-Ala^2-Met^5]enkephalin (Pert *et al.*, 1977) whereas N,N, diallyl-substitution leads to [Leu]enkephalin analogues (particularly N,N-diallyl-[Aib^{2,3}-Leu^5]enkephalin (ICI 174864)) which are pure δ-antagonists (Shaw *et al.*, 1982; Cotton *et al.*, 1984).

Following this line of evidence and with the aim of obtaining a κ-selective antagonist peptide, we have designed and synthesized two N,N-diallyl substituted analogues of DPDYN, N,N-diallyl DPDYN (analogue 1) and N,N-diallyl-[Aib^{2,3}]-DPDYN (analogue 2). In the present study we have examined (i) the binding properties (affinity, selectivity) of these newly designed peptides at the three main (μ, δ, κ) types of opioid binding site, (ii) the sensitivity of their binding to the inhibitory effects of Na^+ ions and of guanine nucleotide (Gpp(NH)p) at both μ- and κ-binding sites and (iii) their biological activity in two pharmacological bioassays for κ-receptors (i.e. guinea-pig ileum (GPI) and rabbit vas deferens (RbVD)).

Methods

Animals

Male Wistar rats weighing about 300 g (C.E. JANVIER, Le Genest, France), New Zealand white rabbits weighing about 1500 g (obtained from a local farm) and tri-coloured guinea-pigs weighing about 300 g (COB-LABO-LAP, Yffiniac, France) were used in this study.

Equilibrium binding studies

Preparation of the crude membrane fractions After decapitation of the animal, the whole brain (minus cerebellum) (rat) or the cerebellum (rabbit, guinea-pig) were rapidly excised and weighed. The crude membrane fractions were then prepared following a modified procedure of Meunier *et al.* (1983) as described by Frances *et al.* (1985). In these conditions, the protein content of the crude membrane fractions (final volume (ml) = 120 × nerve tissue weight (g)) amounted to 0.4–0.7 mg ml^{-1} as determined by the methods of Bradford (1976) using bovine serum albumin as standard.

Inhibition experiments at μ-, δ- and κ-binding sites Equilibrium binding experiments were performed at 25°C for 60 min in Tris-HCl 50 mM, pH 7.4 in the presence of peptidase inhibitors (10^{-5} M bestatin, 10^{-3} M L-Leu-L-Leu and 5×10^{-5} M N-(carboxymethyl)-L-Phe-L-Leu). Each assay mixture (1 ml) contained 0.5 ml of membrane suspension (0.20–0.35 mg ml^{-1} protein final concentration). Binding of 2.5 nM [^{3}H]-DSLET, 1.5 nM [^{3}H]-DAGOL and 0.5 nM [^{3}H]-bremazocine was measured in rat brain, rabbit and guinea-pig cerebellum respectively, (i) in the absence (total binding, in sextuplicate) and presence (non-specific binding, in sextuplicate) of 10 μM levorphanol and (ii) in the presence of increasing concentrations of the unlabelled competing peptide (in triplicate). After incubation, the samples were rapidly filtered through glass microfibre filters (Whatman GF/B) and washed (3 × 3 ml ice cold buffer), using a Millipore model 1225 manifold apparatus. The filters were then dried for 20 min at 100°C and counted for ^{3}H in 3 ml of Ready-solv MP cocktail (Beckman) using a Beckman model LS-3150 P liquid scintillation counter (30–35% efficiency).

Effect of Na^+ ions and Gpp(NH)p on the binding at μ- and κ-sites The equilibrium binding experiments were performed under the same experimental conditions and following the same general procedure as described above. Additional or modified experiments were as follows: binding of 0.3 nM [^{3}H]-diprenorphine was studied in guinea-pig and rabbit cerebellum membranes with and without fixed concentrations of 120 mM NaCl and 50 μM Gpp(NH)p (i) in the absence (total binding, in sextuplicate) or presence (non-specific binding, in sextuplicate) of 10 μM levorphanol and (ii) in the presence of the desired concentrations of unlabelled competing (peptide or alkaloid) drugs (in triplicate). Filtration and washing of the samples as well as counting of the radioactivity were performed as described before.

Biological assays in isolated organ preparations

Myenteric plexus-longitudinal muscle from guinea-pig ileum (GPI) and rabbit vas deferens (RbVD) were prepared according to the procedures described by Gyang & Kosterlitz (1966) and Oka *et al.* (1980), respectively. The tissues were suspended in a 10 ml siliconized organ bath containing Krebs solution at 37°C, oxygenated with 95% O_2 plus 5% CO_2. A cocktail of peptidase inhibitors (30 μM bestatin, 2 mM L-Leu-L-Leu and 0.3 μM thiorphan) was added to the

bathing solutions according to McKnight *et al.* (1983). DPDYN and its N,N-diallyl derivatives were tested for agonist activity by application of cumulative concentrations (from 10^{-10} to 10^{-5} M) of each peptide into the bath. Potency of an agonist was assessed by measuring the IC_{50} value (concentration of a peptide inhibiting by 50% the response to electrical field stimulation). The N,N-diallyl-substituted peptides were tested for antagonism against DPDYN (in GPI and RbVD) and against DAGOL (in GPI) by introducing the analogue into the bath at least 10 min before testing the activity of the agonist. Activities of the antagonists were expressed in terms of Ke as defined by Kosterlitz & Watt (1968): $Ke = a/(DR - 1)$ where a is the molar concentration of the antagonist and DR is the ratio of the concentration of the agonist required to depress the twitch to the same extent in the presence or absence of a given concentration of the antagonist.

Labelled probes

[15,16(n)-^{3}H]-diprenorphine (0.92–1.85 TBq mmol^{-1}) and [^{3}H]-RX783006 ([3,5-^{3}H]-Tyr-D-Ala-Gly-MePhe-NH$(CH_2)_2$OH, [^{3}H]-DAGOL, 1.11–2.22 TBq mmol^{-1}), Amersham International (Amersham, U.K.). [9, ^{3}H(N)]-(−)-bremazocine (0.55–1.11 TBq mmol^{-1}) and [3,5-^{3}H(N)]Tyr-D-Ser-Gly-Phe-Leu-Thr ([^{3}H]-DSLET, 0.92–1.47 TBq mmol^{-1}), New England Nuclear Chemicals (Dreieich, F.R.G.) were used.

Unlabelled chemicals

Diprenorphine hydrochloride, Reckitt and Colman (Kingston, U.K.); naloxone hydrochloride, Endo Laboratories (Garden City, NY, U.S.A.); DAGOL, Cambridge Research Ltd. (Harston, U.K.), guanyl-5′-yl imidodiphosphate (Gpp (NH)p), sodium salt, Sigma Chemical Co. (St Louis, MO, U.S.A.). MR2266 ((−)-(1**R**, 5**R**, 9**R**)-5,9-diethyl-2-(3-furylmethyl)2′-hydroxy-6,7-benzomorphan) and U-50, 488H (*trans*-(+)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolydinyl)cyclohexyl]benzeneacetamide methane sulphonate) were kindly supplied by Boehringer (Ingelheim, F.R.G.) and the Upjohn Company (Kalamazoo, MI, U.S.A.), respectively.

Peptides

[D-Pro^{10}]dynorphin(1–11): TyrGlyGlyPheLeuArg-ArgIleArg D-ProLys (DPDYN) was synthesized following the solid-phase procedure as described elsewhere (Gairin *et al.*, 1986). Before synthesis of analogues 1 and 2, the initial compound N,N-diallyl (O-benzyl)tyrosine was obtained from the reaction of a large excess of allyl bromide with (O-benzyl)tyrosine methyl ester in presence of N,N-diisopropyl ethylamine in toluene followed by hydrolysis of the methyl ester. The N,N-diallyl-tyrosyl residue was checked for stability under HF cleavage conditions and the synthesis of the analogues was then performed following the solid-phase procedure as mentioned above. The peptides were purified by ion exchange chromatography and checked for purity by t.l.c. and reverse-phase h.p.l.c. and characterized by amino acid analysis, ^{1}H n.m.r. and fast atom bombardment mass spectrometries.

Peptidase inhibitors

Bestatin (Cambridge Research Biochemicals, Cambridge, U.K.), L-Leu-L-Leu (Bachem AG, Budendorf, Switzerland), thiorphan (Peninsula Inc., Belmont, CA, U.S.A.) and N-carboxymethyl-L-Phe-L-Leu (synthesized by P. Alvinerie) were used.

Results

Inhibitory effects of DPDYN and its N,N-diallyl substituted analogues at μ-, δ- and κ-binding sites

Substitution on the tyrosyl nitrogen atom may affect the opioid peptide-receptor recognition process. As shown in Table 1, replacing the two hydrogen atoms of the primary amino group by two allyl groups leads to a dramatic fall in affinity of the peptide (analogue 1) at μ-, δ- and κ-sites (54, 47 and 770 fold decrease, respectively). As a consequence, the κ-selectivity of DPDYN ($\kappa/\mu = 92$ and $\kappa/\delta = 278$) is greatly reduced but not completely lost ($\kappa/\mu = 7$ and $\kappa/\delta = 17$). In analogue 2 where α-aminoisobutyric acid (Aib) is substituted for the glycyl residues in position 2 and 3 no further significant decrease of affinity was observed at κ-sites while somewhat lower affinities were measured at both μ and δ sites. Considering the measured inhibitory effects, all the corresponding calculated slope factors (shown in parentheses in Table 1) are very close to unity, indicating a homogeneous population of labelled sites and a competitive inhibition at these sites.

Sensitivity to Na^+ ions and guanine nucleotide of the binding of DPDYN and its analogues at μ- and κ-binding sites

The binding sensitivity of DPDYN and of its N,N-diallyl-substituted analogues to Na^+ ions and Gpp(NH)p was studied by measuring the inhibitory effects of the peptides on the binding of the labelled

Table 1 Apparent affinity (K_i, nM) of [D-Pro^{10}]dynorphin A (1–11) (DPDYN) and of its related N,N-diallyl-substituted analogues at μ-, δ- and κ-binding sites

Peptide	*μ-sites*	*δ-sites*	*κ-sites*
DPDYN	2.5 ± 0.7	7.5 ± 1.3	0.027 ± 0.007
	(0.91 ± 0.05)	(1.19 ± 0.15)	(1.03 ± 0.04)
Analogue 1: N,N-diallyl-DPDYN	135 ± 22	354 ± 79	20.7 ± 5.0
	(1.24 ± 0.05)	(0.92 ± 0.07)	(0.82 ± 0.01)
Analogue 2: N,N-diallyl-[Aib^{2,3}]-DPDYN	299 ± 30	530 ± 90	18.9 ± 3.2
	(0.91 ± 0.05)	(1.01 ± 0.10)	(0.84 ± 0.09)

The values are the mean ± s.e.mean of at least three independent experiments performed in triplicate. Binding experiments were carried out at 25°C for 60 min in the presence of peptidase inhibitors (10^{-5} M bestatin, 10^{-3} M L-Leu-L-Leu and 5×10^{-5} M N-(carboxymethyl)-L-Phe-L-Leu). The inhibitory effects (K_i, nM) of the peptides were measured against [^{3}H]-DAGOL (1.5 nM) in rabbit cerebellum (μ-sites), [^{3}H]-DSLET (2.5 nM) in rat brain (δ-sites) and [^{3}H]-bremazocine (0.5 nM) in guinea-pig cerebellum (κ-sites) membranes. K_i values were calculated from the equation of Cheng & Prusoff using 1.6, 1.9 and 0.07 nM for the K_D of the tritiated DAGOL, DSLET and bremazocine, respectively. The apparent Hill coefficients are given in parentheses.

antagonist [^{3}H]-diprenorphine in the presence or absence of the two allosteric effectors in rabbit and guinea-pig cerebellum membranes. These two preparations contain a high proportion (>80%) of μ (Meunier *et al.*, 1983)- and of κ (Robson *et al.*, 1984)-opioid sites, respectively, and may thus be considered as ideal tissues for the biochemical studies on molecular mechanisms that may operate at these types of site. Na^+ ions and Gpp(NH)p, a non-hydrolysable analogue of GTP, were used in combination at 120 mM and 50 μM, respectively. Before testing the binding sensitivity of the peptides, we verified that the presence of 120 mM NaCl + 50 μM Gpp(NH)p did not significantly affect the binding of [^{3}H]-diprenorphine (0.3 nM) in the two preparations (see legends of Tables 2 and 3), as previously demonstrated (Frances *et al.*, 1985). In the rabbit cerebellum, the μ-selective agonist DAGOL and the antagonists diprenorphine (non-selective) and naloxone (comparatively μ-selective) were used as reference compounds. As shown in Table 2, binding at μ-sites of the μ-agonist DAGOL was greatly inhib-

Table 2 Effects of 120 mM NaCl + 50 μM Gpp(NH)p on the binding at μ-sites of [D-Pro^{10}]dynorphin A (1–11) (DPDYN) and of its N,N-diallyl-substituted analogues, the μ-selective agonist DAGOL, and of the antagonists diprenorphine and naloxone

	Opioid	120 mM NaCl + 50 μM Gpp(NH)p (−)	(+)	*Ratio* $IC_{50}(+)/IC_{50}(-)$
μ-Agonist	DAGOL	9.6 ± 2.9	2190 ± 230	230
		(0.48 ± 0.06)	(0.66 ± 0.03)	
Antagonists	Diprenorphine	0.65 ± 0.09	0.58 ± 0.03	0.9
		(1.05 ± 0.08)	(1.06 ± 0.02)	
	Naloxone	8.3 ± 3.5	4.3 ± 0.5	0.5
		(1.02 ± 0.06)	(1.08 ± 0.05)	
Peptides	DPDYN	2.6 ± 0.9	1178 ± 208	450
		(0.84 ± 0.09)	(0.78 ± 0.08)	
	N,N-diallyl-DPDYN	193 ± 11	297 ± 8	1.5
		(0.77 ± 0.02)	(0.83 ± 0.03)	
	N,N-diallyl-[Aib^{2,3}]-DPDYN	262 ± 17	292 ± 20	1.1
		(0.81 ± 0.02)	(0.77 ± 0.08)	

The inhibitory effects (IC_{50}, nM) of the drugs were measured at 25°C in the presence of peptidase inhibitors against [^{3}H]-diprenorphine (0.3 nM) in rabbit cerebellum membranes (μ-sites) in the absence (−) and in the presence (+) of the combination of the two allosteric modulators. The binding of the labelled probe amounted to 160.3 ± 21.7 and 219.5 ± 15.9 fmol mg^{-1} protein in the absence and in the presence of added effectors, respectively. The values are the mean ± s.e. of at least three independent experiments performed in triplicate, slope factors are indicated in parentheses. DAGOL = Tyr-D-Ala-Gly-MePhe-NH(CH$_2$)$_2$OH.

ited in the presence of the two allosteric effectors (IC_{50} ratio = 230) while binding of the two antagonists was not (IC_{50} ratios close to 1). These observations are in complete agreement with data obtained previously (Frances *et al.*, 1985). Under the same experimental conditions, we found binding of DPDYN at μ-sites to be highly sensitive to Na^+ ions and Gpp(NH)p (IC_{50} ratio = 450). In contrast, we observed no significant change in the inhibitory effects of either N,N-diallyl DPDYN or N,N-diallyl [$Aib^{2,3}$] DPDYN (IC_{50} ratio values of 1.5 and 1.1, respectively). These IC_{50} ratios are very close to those observed with diprenorphine or naloxone suggesting that the two diallyl-substituted peptides could be antagonists at the μ-receptor.

In guinea-pig cerebellum membranes, we used the κ-selective agonist U-50488H and the antagonists diprenorphine (non-selective) and MR2266 (relatively κ-selective) as standard reference compounds. As expected, we found (Table 3) that 120 mM Na^+ + 50 μM Gpp(NH)p inhibited the binding of the agonist U-50, 488H at κ sites, while binding of the antagonists was affected very little, or not at all (diprenorphine, IC_{50} ratio = 1.1; MR2266, IC_{50} ratio = 2.2). With the peptides, we observed a dramatic fall in the κ-binding affinity of DPDYN when measured in the presence of Na^+ and Gpp(NH)p (IC_{50} ratio = 140); in contrast, as observed in rabbit cerebellum, N,N-diallyl-substitution led to a complete loss of sensitivity of the analogues to Na^+ ions and Gpp(NH)p at the κ-sites of the guinea-pig cerebellum (IC_{50} ratio values lower than 2).

Furthermore, it must be noted that in both the rabbit and guinea-pig cerebellum, the values for slope factor for the displacements by the antagonists and the diallyl peptides were close to unity, while those for the agonists DAGOL, U-50,488H or DPDYN were significantly lower than 1. These findings could indicate that antagonists and agonists are competing with [^{3}H]-diprenorphine at respectively, one (antagonist), or two (agonist + antagonist) states of opioid binding sites.

Together these data suggested that N,N-diallyl-DPDYN and N,N-diallyl [$Aib^{2,3}$] DPDYN might have antagonist properties at both μ- and κ-opioid binding sites.

Antagonist effects of the diallyl substituted analogues of DPDYN in isolated organ preparations

The biological activities of the N,N-diallyl analogues of DPDYN were measured in preparations of guinea-pig ileum (GPI) and rabbit vas deferens (RbVD). As shown in Table 4, when tested for agonist activity, none of the substituted peptides was able to inhibit the electrically-induced contractions of either GPI or RbVD at bath concentrations of up to 10 μM. This lack of agonist activity at both μ- and κ-opioid receptors in isolated tissues correlated well with the lack of sensitivity to sodium ions and guanine nucleotide observed in binding studies. Analogues 1 and 2 were then tested for antagonist activity at κ-receptors against DPDYN in GPI and RbVD and at μ-receptors against DAGOL in GPI.

Table 3 Effects of 120 mM NaCl + 50 μM Gpp(NH)p on the binding at κ-sites of [D-Pro^{10}]dynorphin A (1–11) (DPDYN) and of its N,N-diallyl-substituted analogues, the κ-selective agonist U-50, 488H and of the antagonists diprenorphine and MR2266

	Opioid	120 mM NaCl + 50 μM Gpp(NH)p (−)	(+)	*Ratio* $IC_{50}(+)/IC_{50}(-)$
κ-Agonist	U-50,488H	8.4 ± 0.5 (0.59 ± 0.07)	214 ± 10 (0.53 ± 0.04)	25
Antagonists	Diprenorphine	0.41 ± 0.15 (0.80 ± 0.09)	0.44 ± 0.13 (0.87 ± 0.10)	1.1
	MR2266	0.45 ± 0.16 (0.75 ± 0.10)	0.96 ± 0.38 (0.76 ± 0.12)	2.1
Peptides	DPDYN	0.22 ± 0.07 (0.67 ± 0.12)	31 ± 5 (0.46 ± 0.02)	140
	N,N-diallyl-DPDYN	103 ± 38 (0.98 ± 1.15)	154 ± 44 (0.97 ± 0.18)	1.5
	N,N-diallyl-[$Aib^{2,3}$]-DPDYN	79 ± 26 (0.53 ± 0.06)	147 ± 39 (0.75 ± 0.09)	1.9

The inhibitory effects (IC_{50}, nM) of the drugs were measured at 25°C in the presence of peptidase inhibitors against [^{3}H]-diprenorphine (0.3 nM) in guinea-pig cerebellum membranes (κ-sites) in the absence (−) and in the presence (+) of the allosteric modulators. The binding of the labelled probe amounted to 91.7 ± 12.4 and 77.3 ± 9.0 fmol mg^{-1} protein (n = 6) in the absence and in the presence of added effectors, respectively. The values are the mean ± s.e. of at least three independent experiments performed in triplicate, slope factors are indicated in parentheses.

Table 4 Biological activities of [D-Pro^{10}] dynorphin A (1–11) (DPDYN) and of its N,N-diallyl-substituted analogues on the guinea-pig isolated ileum (GPI) and rabbit vas deferens (RbVD) preparations

		Rabbit vas deferens		*Guinea-pig ileum*		
	Peptide	IC_{50} (nM)	*Ke* (nM)/*DPDYN*	IC_{50} (nM)	*Ke* (nM)/*DAGOL*	*Ke* (nM)/*DPDYN*
	DPDYN	17 ± 5 ($n = 7$)	—	13 ± 4 ($n = 5$)	—	—
Analogue 1	N,N-diallyl-DPDYN	>10,000	97 ± 22 ($n = 7$)	>10,000	228 ± 38 ($n = 7$)	188 ± 32 ($n = 5$)
Analogue 2	N,N-diallyl-[Aib^{2,3}]-DPDYN	>10,000	131 ± 27 ($n = 7$)	>10,000	248 ± 51 ($n = 9$)	130 ± 44 ($n = 7$)

The values are the mean ± s.e.mean; the number of observation (n) is given in parentheses. The Krebs solution contained the following peptidase inhibitors: 30 μM bestatin, 2 mM L-Leu-L-Leu and 0.3 μM thiorphan. Bath temperature was 37°C. The antagonist activities (Ke, nM) of the N,N-diallyl analogues of DPDYN were measured in RbVD against DPDYN and in GPI against DPDYN and DAGOL. In absence of antagonist the inhibitory effect of DAGOL in GPI was 17 ± 7 nM ($n = 7$).

In the absence of antagonist, the IC_{50} of the μ-selective agonist DAGOL in GPI was 17 ± 7 nM ($n = 7$); the IC_{50}s of the κ-selective agonist DPDYN in GPI and RbVD were respectively 13 ± 4 nM ($n = 5$) and 17 ± 5 nM ($n = 7$), in agreement with data obtained previously (Gairin *et al.*, 1986). Against DPDYN, N,N-diallyl [Aib^{2,3}]DPDYN displayed very similar antagonist properties in GPI and RbVD (130 ± 44 nM and 131 ± 27 nM for the respective Ke values), but the affinity was somewhat greater in RbVD than in GPI with N,N-diallyl-DPDYN (188 ± 32 nM in GPI and 97 ± 22 nM in RbVD). At μ-receptors in GPI, analogues 1 and 2 showed similar affinities when tested against DAGOL; the observed values for Ke were 228 ± 38 nM and 248 ± 51 nM, respectively. Thus, the absolute values for affinity of the antagonists and the observed greater affinity for κ-receptors agree well with the values obtained from the binding assays.

Discussion

In the search for selective antagonists for κ-sites, we have designed two analogues of the κ-selective opioid peptide DPDYN with N,N-diallyl substitution, by analogy with δ-selective antagonist peptides derived from [Leu]enkephalin (Shaw *et al.*, 1982; Cotton *et al.*, 1984). As postulated for N-allyl substituted opiate alkaloid antagonists, it is thought that N,N-diallyl-substitution may induce a specific antagonist conformation to the opioid peptides. However, the N-terminal tyrosyl residue plays an essential role in the binding of opioid peptides to their receptors and the steric hindrance resulting from incorporation of one or two bulky allyl groups at the nitrogen atom can be expected to alter significantly the binding properties (i.e. affinity and selectivity) of the peptide. In accordance with data obtained previously on N-allyl- and N,N-diallyl-enkephalin derivatives (Pert *et al.*, 1977; Corbett *et al.*, 1984), a great loss of affinity was observed for the N,N-diallyl-derivatives of DPDYN, the greatest effect occurring at the preferred (κ) binding site. Interestingly, a further structural modification by replacement of Gly by Aib at positions 2 and 3 in the sequence of DPDYN did not affect the affinity.

It has been demonstrated (Frances *et al.*, 1985) that Na^+ ions and Gpp(NH)p in combination (120 mM NaCl + 50 μM Gpp(NH)p) selectively inhibit equilibrium binding of agonists but not of antagonists in cerebellum membranes of the rabbit (μ-sites) and of the guinea-pig (κ-sites). Thus, competition experiments against [^{3}H]-diprenorphine binding in these two specific preparations, in the presence or absence of the two allosteric effectors, appears to be of some utility for the *in vitro* determination of the binding properties of novel κ- or μ-selective ligands. DPDYN, which acts as a typical κ-agonist in isolated organ preparations (Gairin *et al.*, 1986) displays high sensitivity to allosteric effectors at both κ- and μ-sites, the greater magnitude of the effect being observed at μ-sites. Considering the κ-selectivity of DPDYN, such a result could, in a way, be unexpected since it has been proposed that the greater magnitude in affinity change of an agonist is expected to occur at its preferred site (Frances *et al.*, 1985). However the fact that DPDYN as well as the other dynorphin peptides also show high affinity for μ-receptors at which they act as agonists may explain this observation. At the κ-binding sites, Na^+ ions and Gpp(NH)p appear to be less effective against U-50,488H than against DPDYN. As far as this observation is significant, one can hypothesize that the inhibitory effect of the allosteric effectors may vary depending on the chemical nature (non peptide vs peptide) of the agonist. As was recently

suggested for dynorphin (1–8) and (1–9), it seems unlikely that a change in the rate of enzymatic degradation of the peptides due to the change in ionic composition would be sufficient to explain such a result (Paterson *et al.*, 1986; Kosterlitz *et al.*, 1987).

On the other hand, of particular interest is the lack of effect of NaCl and Gpp(NH)p on binding of the N,N-diallyl-subsituted analogues of DPDYN at κ-sites in guinea-pig cerebellum. In this preparation, the binding characteristics of the peptides are comparable to those for the non-peptide antagonists diprenorphine and MR 2266. One can therefore postulate that N,N-diallyl-DPDYN and N,N-diallyl-[Aib^{2,3}]-DPDYN are endowed with antagonist properties at κ-opioid sites. The same conclusion can be drawn for μ-sites, since the binding of the analogues appears to be as insensitive to the effects of the allosteric modulators as is the binding of the non-peptide antagonists diprenorphine and naloxone in rabbit cerebellum membranes.

In *in vitro* pharmacological bioassays in guinea-pig myenteric plexus and rabbit vas deferens preparations, the complete lack of agonist activity and the affinity as antagonists of the two N,N-diallyl analogues of DPDYN closely correlates with the lack of sensitivity to Na^+ ions and Gpp(NH) observed in binding studies. From bioassay experiments, the two substituted analogues of DPDYN appear to be of approximately equal affinity. These results indicate that antagonism at κ-, and to a lesser extent at μ-receptors is induced in DPDYN by N,N-diallyl-substitution of the tyrosyl residue and that the nature of the peptide chain (Gly vs Aib) does not influence the antagonist potency of the peptide. The latter finding is in contrast to those obtained for the diallyl substituted δ-antagonist analogues of leucine enkephalin (Shaw *et al.*, 1982; Corbett *et al.*, 1984; Cotton *et al.*, 1984). Unfortunately, as shown in binding studies, N,N-diallyl substitution also leads to a dramatic fall in both affinity and selectivity (κ vs μ) of the peptide.

In conclusion, the findings underline the promise of this approach of diallyl-substitution in selective agonists for the design of selective antagonists for opioid receptors. However, it can be concluded that, while selective antagonists for the δ-receptor have been obtained in this way, the goal of selectivity for κ- over μ-receptors is more elusive, perhaps because of a similarity of tertiary structure in the conformation of the putative antagonist states of the receptor molecules (Frances *et al.*, 1985). It is encouraging, therefore, that recent findings (Portoghese & Takemori, 1985; Portoghese *et al.*, 1987) suggest that antagonist selectivity for the κ-receptor may not be unachievable.

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