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Pre-incubation of guinea-pig myenteric plexus with β-funaltrexamine: discrepancy between binding assays and bioassays

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1 The acute effects of β-funaltrexamine and the effects of pre-incubation with this compound were examined in five *in vitro* assay tissues and in selective binding assays in homogenates of guinea-pig brain and myenteric plexus.

2 In competitive displacement assays with selective ligands, β-funaltrexamine had highest affinity for the μ-binding site in the myenteric plexus and brain of guinea-pig. Its affinity for the κ-site was about 15% of that for the μ-site.

3 Pre-incubation of the assay tissues with β-funaltrexamine caused an increase in the IC_{50} values of μ- and δ-receptor agonists but not of κ-agonists. Although in bioassays on the myenteric plexus-longitudinal muscle preparation of the guinea-pig, the IC_{50} value of the μ-receptor ligand [D-Ala^2, MePhe^4, Gly-ol^5] enkephalin was increased up to 124 fold, its binding at the μ-site in homogenates of the preparation was not affected by this treatment.

4 These findings indicate that the effects of pre-incubation with β-funaltrexamine on agonist potency of the μ-receptor ligand are due to an interference with the coupling mechanism between the μ-binding site and the effector system.

Introduction

It is now generally accepted that opioids interact with at least three different receptors, μ, δ and κ. Although selective ligands are available for each receptor type, it would be useful to block irreversibly a single receptor type and thus facilitate the investigation of the mode of action of opioids in tissues containing a heterogeneous population of opioid receptors.

Recently β-funaltrexamine, the fumaramate methyl ester derivative of the opioid antagonist naltrexone, has been proposed by Portoghese and his group to be such a ligand (Portoghese *et al.*, 1980; Ward *et al.*, 1982b). It has been shown that, in acute experiments, it is an agonist in the guinea-pig myenteric plexus-longitudinal muscle preparation. However, exposure of this tissue to β-funaltrexamine for periods of 30 to 90 min causes an irreversible decrease in the agonist potencies of morphine and normorphine, which interact with μ-receptors, without altering the agonist potency of ethylketazocine, which interacts with κ-receptors (Takemori *et al.*, 1981; Huidobro-Toro *et al.*, 1982; Ward *et al.*, 1982b).

The effects on binding have been presented in the form of an abstract which showed that β-funaltrexamine binds to those sites with which μ- and κ-receptor agonists interact in mouse brain and that a portion of this binding is irreversible (Ward *et al.*, 1980). Furthermore, pre-incubation of rat brain homogenates with β-funaltrexamine reduces the maximal binding of [^{3}H]-naloxone (Rothman *et al*, 1983).

We have extended these observations on the irreversible effects of β-funaltrexamine in isolated tissues using agonists highly selective for the μ, δ- or κ-receptor. The assays were the guinea-pig myenteric plexus-longitudinal muscle preparation which has both μ- and κ-receptors (Hutchinson *et al.*, 1975; Chavkin & Goldstein, 1981) and the vasa deferentia of the hamster having only δ-receptors (McKnight *et al.*, 1984a), the rabbit having only κ-receptors (Oka *et al.*, 1981), the mouse having all three receptors (Lord *et al.*, 1977) and the rat having μ- and possibly ε-receptors (Wüster *et al.*, 1979; Gillan *et al.*, 1981). In addition to the bioassays, the effects of β-funaltrex-

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amine on binding at the μ-, δ- and κ-sites were examined in homogenates of the brain and of the myenteric plexus of the guinea-pig. Some of the results have been presented previously (Corbett *et al.*, 1984b; McKnight *et al.*, 1984b).

Methods

Bioassays

The myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum and the vasa deferentia of the mouse, rat, rabbit and hamster were set up for field stimulation as described previously (McKnight *et al.*, 1983; 1984a). The potencies of agonists were obtained from dose-response curves, with the exception of β-funaltrexamine in the mouse vas deferens where the IC_{50} was determined by a 'single-dose' method (Kosterlitz & Watt, 1968) since β-funaltrexamine has both agonist and antagonist actions in this tissue. The antagonist equilibrium dissociation constant (K_e, nM) of naloxone was determined in the myenteric plexus preparation against [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin as agonist by the method of Arunlakshana & Schild (1959).

Binding assays

In binding assays in homogenates of guinea-pig brain the μ-site was selectively labelled with [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5] enkephalin (1 nM), the δ-site selectively with [^{3}H]-[D-Ala^2, D-Leu^5]enkephalin (0.7 nM) in the presence of 30 nM unlabelled [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin to suppress μ-binding and the κ-site selectively with [^{3}H]-(−)-bremazocine (0.1 nM) in the presence of 100 nM each of unlabelled [D-Ala^2, MePhe^4, Gly-ol^5] enkephalin and [D-Ala^2, D-Leu^5]enkephalin to suppress μ- and δ-binding (Corbett *et al.*, 1984a). The inhibition constants (K_i, nM) were calculated by the method of Cheng & Prusoff (1973).

Homogenates of guinea-pig myenteric plexus-longitudinal muscle were prepared by a modification of the method of Leslie & Kosterlitz (1979). Strips of myenteric plexus-longitudinal muscle were finely chopped in 50 mM Tris buffer (pH 7.4 at 0°C) and diluted to a concentration of 100 mg tissue ml^{-1}. The tissue was homogenized with an Ultra-Turrax homogenizer followed by 10 strokes of a teflon pestle in a glass homogenizer. This homogenate was filtered through a fine curtain net to remove any residual large particles. The filtrate was centrifuged at 49,000 *g* for 10 min, the pellet resuspended in Tris buffer and kept overnight in a refrigerator at 4°C. The stored homogenate was then centrifuged, the pellet resuspended in Tris buffer (pH 7.4 at 37°C), incubated at 37°C for 30 min and centrifuged again. For the binding assay, 0.96 ml of the final homogenate (100 mg tissue ml^{-1}) was used. The volume was made up to 1 ml with solutions of the inhibitory ligand and the labelled ligand. The samples were incubated for 40 min at 25°C and then filtered as described previously (Magnan *et al.*, 1982). In competition experiments, the μ-binding site was labelled with [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin (6 nM) and the κ-site with [^{3}H]-(−)-bremazocine (0.5 nM) in the presence of 250 nM each of unlabelled [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin and [D-Ala^2, D-Leu^5]enkephalin. The binding capacity and the equilibrium dissociation constant (K_D) were determined for [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin, and for [^{3}H]-(−)-bremazocine in the presence and absence of 250 nM each of unlabelled [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin and [D-Ala^2, D-Leu^5]enkephalin.

Pre-incubation of isolated tissues with β-funaltrexamine

The effects of pre-incubation with β-funaltrexamine on the potency of opioid agonists were examined in the various pharmacological preparations as follows. After the determination of the IC_{50} of an agonist, the tissues were incubated with β-funaltrexamine at various concentrations for 30, 60 or 90 min. To remove residual β-funaltrexamine, the preparations were then washed for at least 60 min with drug-free Krebs solution, by overflow at 1 ml min^{-1}, and the potency of the agonist was again determined.

Pre-incubation of tissue homogenates with β-funaltrexamine

Guinea-pig brains were homogenized in Tris buffer (pH 7.4 at 0°C) and centrifuged at 49,000 *g* for 10 min; the pellet was resuspended in Tris buffer (pH 7.4 at 37°C). Tris buffer (100 μl) or β-funaltrexamine in Tris buffer was added to 20 ml aliquots of homogenate and the samples incubated at 37°C for up to 2 h. At the end of the incubation, the samples were centrifuged, the pellet resuspended in drug-free Tris buffer, incubated at 37°C for 15 min and recentrifuged. This washing procedure was repeated. Binding at the μ-, δ- and κ-sites was measured in the final homogenate.

Homogenates of guinea-pig myenteric plexus, which had been stored overnight at 4°C, were centrifuged and the pellets resuspended in Tris buffer (pH 7.4 at 37°C). Solutions of Tris buffer or β-funaltrexamine in Tris buffer were added to aliquots of homogenate which were incubated at 37°C for 30 or 60 min. The homogenate was then washed as described for brain homogenates and the binding at the μ- and κ-sites was determined.

Labelled ligands

The labelled ligands used were [^{3}H]-[D-Ala^2, D-Leu^5]enkephalin (43–56 Ci mmol^{-1}) and [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin (35 Ci mmol^{-1}) from Amersham International and [^{3}H]-(−)-bremazocine (24 Ci mmol^{-1}) from Dr D. Römer, Sandoz. The tritiated ligands were of a purity of >95%; this was achieved, when necessary, by reverse phase h.p.l.c. on a μ-Bondapak C_{18} column.

Drugs

The drugs used were obtained from the following sources: [D-Pen^2, D-Pen^5]enkephalin (Dr R. Cotton, ICI), [D-Ala^2, D-Leu^5]enkephalin and β_h-endorphin (Cambridge Research Biochemicals), [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin (Dr D. Römer, Sandoz), morphine hydrochloride (MacFarlan Smith), U-50,488H (*trans*-3,4-dichloro-N-methyl-N-(2-(1-pyrrolidinyl)cyclohexyl)-benzeneacetamide] (Dr R. Lahti, The Upjohn Company), (−)-ethylketazocine (Dr W. Michne, Sterling-Winthrop), β-funaltrexamine (Dr R. Cotton, ICI), diprenorphine (Reckitt & Colman), Mr 2266 [(−)-5,9-diethyl-2'-hydroxy-2-3-furylmethyl)-6,7-benzomorphan] (Dr H. Merz, Boehringer-Ingelheim) and naloxone hydrochloride (Endo Laboratories).

Stock solutions were prepared in water and stored at −25°C. Dilutions for binding assays were made with 50 mM Tris buffer solutions at pH 7.4 and for bioassays with Krebs solution.

Results

Agonist actions of β-funaltrexamine in acute experiments

β-Funaltrexamine inhibited the electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation of the guinea-pig; the IC_{50} was 4.35 ± 0.75 nM (n = 7) which agrees with the findings of Takemori *et al.* (1981). It was much less active in the vasa deferentia of the mouse and the rabbit, where the IC_{50} values were 190 ± 35 nM (n = 4) and 990 ± 140 nM (n = 11) respectively. β-Funaltrexamine had no agonist activity in the vas deferens of the hamster and of the rat.

Effects of pre-incubation with β-funaltrexamine on the potency of agonists in the guinea-pig myenteric plexus

Pre-incubation of the myenteric plexus with 100 nM β-funaltrexamine for 30 min led to an increase in the IC_{50} value for morphine, confirming the findings of Takemori *et al.* (1981). However, although morphine interacts mainly with μ-receptors, it has significant affinities for the δ- and κ-receptors (Magnan *et al.*, 1982). It was therefore important to extend these observations with the more selective μ-agonist [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin. The ratio of the IC_{50} after pre-incubation with β-funaltrexamine to the IC_{50} before treatment was greater for the peptide analogue than for morphine. In contrast, the IC_{50} value of the selective κ-agonist, U-50,488H, was not altered (Table 1).

When the duration of the pre-incubation with 100 nM β-funaltrexamine was progressively increased from 0 min to 90 min, the IC_{50} values of [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin increased from a mean of 9 nM to 1060 nM; when the myenteric plexus was pre-incubated for 30 min with 1000 nM β-funaltrexamine the initial and final IC_{50} values were 7 nM and 96 nM (Table 2).

The antagonist equilibrium dissociation constant (K_e, nM) of naloxone against the agonist [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin was not altered by pre-incubation of the myenteric plexus with 100 nM β-funaltrexamine for 30 or 60 min but it was significantly increased from 1.9 nM to 3.1 or 4.7 nM when the pre-incubation was with either 100 nM β-funaltrexamine for 90 min or 1000 nM β-funaltrexamine for 30 min (Table 2). In each case, the slope of the Schild plot was close to unity. The increase in the K_e may, at least partly, be due to the fact that high concentrations of

Table 1 The effects of pre-incubation with β-funaltrexamine on the agonist potencies of opioids in the myenteric plexus-longitudinal muscle preparation of the guinea-pig

	Agonist potency (IC_{50}, nM)		
	Before β-FNA	*After β-FNA*	*Mean dose-ratio*
[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin	6.5 ± 0.85	80 ± 20	11.9 ± 2.4 (9)
Morphine	130 ± 24	830 ± 159	7.3 ± 1.4 (6)
U-50,488H	1.17 ± 0.62	1.63 ± 0.97	1.3 ± 0.15 (4)

The values are the means ± s.e.mean; the number of observations is given in parentheses. Tissues were pre-incubated with 100 nM β-funaltrexamine (β-FNA) for 30 min, then washed for 60 min with drug-free Krebs solution. Dose-ratio is the ratio of IC_{50} after pre-incubation with β-FNA to IC_{50} before treatment.

Table 2 The effects of pre-incubation with β-funaltrexamine on the agonist activity of [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin in the myenteric plexus-longitudinal muscle preparation of the guinea-pig

Pre-incubation	[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin (IC_{50}, nM) Before β-FNA	After β-FNA	Mean dose-ratio	Naloxone (K_e, nM)
(a) β-FNA 100 nM				
0 min	7.6 ± 1.4			1.89 ± 0.29 (3)
30 min	6.4 ± 0.33	95.2 ± 42.9	14.0 ± 5.6	1.80 ± 0.21 (4)
60 min	13.5 ± 1.0	840 ± 160	61.6 ± 11.5	2.82 ± 0.70 (3)
90 min	8.3 ± 0.87	1060 ± 340	124 ± 29.3	3.07 ± 0.19 (3)*
(b) β-FNA 1000 nM				
30 min	7.0 ± 3.2	96.3 ± 17.7	21.0 ± 8.1	4.68 ± 0.70 (3)*

The values are the means ± s.e.mean; the number of observations is given in parentheses. Individual preparations of myenteric plexus were exposed to β-funaltrexamine (β-FNA) for 30, 60 or 90 min and then washed for 60 min with drug-free Krebs solution. Dose ratio is the ratio of IC_{50} after preincubation with β-FNA to IC_{50} before treatment. *$P < 0.05$ compared with untreated control.

[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin can interact with the κ-receptor.

It has to be noted that if the pre-incubation was with either 1000 nM β-funaltrexamine for 60 min or with 3000 nM for 30 min, the contractions of the myenteric plexus were less than half of their initial magnitude, even after washing for 120 min. Therefore, the agonist potency of [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin was not determined.

Effects of pre-incubation with β-funaltrexamine on the potency of agonists in the vasa deferentia of the mouse, hamster, rabbit and rat

β-Funaltrexamine had a weak agonist action in the vasa deferentia of the mouse and the rabbit but was inactive in the hamster and the rat. In all four species the size of the electrically-evoked contractions was not altered after pre-incubation with 100 nM β-funaltrexamine for 30 min followed by washing for 60 min with drug-free Krebs solution.

The effects of pre-incubation with 100 nM β-funaltrexamine for 30 min were determined on δ-receptors in the vas deferens of the mouse and of the hamster; the agonists were [D-Ala^2, D-Leu^5]enkephalin and the highly selective δ-ligand [D-Pen^2, D-Pen^5]enkephalin. After pre-incubation, the IC_{50} values of [D-Ala^2, D-Leu^5]enkephalin and of [D-Pen^2, D-Pen^5]enkephalin were increased in the vasa deferentia of both species (Table 3). When the duration of pre-incubation of the hamster vas deferens with β-funaltrexamine is increased from 30 to 60 min neither [D-Ala^2, D-Leu^5]enk-

Table 3 The effects of pre-incubation with β-funaltrexamine on the agonist potencies of opioids in the vasa deferentia of the mouse, hamster, rabbit and rat

	Agonist potency (IC_{50}, nM) Before β-FNA	After β-FNA	Mean dose-ratio
Mouse vas deferens			
[D-Pen^2, D-Pen^5]enkephalin	4.98 ± 0.55	17.2 ± 4.1	3.3 ± 0.78 (8)
[D-Ala^2, D-Leu^5]enkephalin	0.42 ± 0.06	1.54 ± 0.13	3.8 ± 0.47 (4)
Hamster vas deferens			
[D-Pen^2, D-Pen^5]enkephalin	190 ± 11	1460 ± 230	7.6 ± 0.99 (4)
[D-Ala^2, D-Leu^5]enkephalin	20.8 ± 5.7	93 ± 29	4.4 ± 0.53 (3)
Rabbit vas deferens			
U-50,488H	84 ± 14	38 ± 5.7	0.48 ± 0.08 (4)
Ethylketazocine	12.1 ± 3.35	9.6 ± 1.84	0.86 ± 0.18 (4)
Rat vas deferens			
[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin	350 ± 40	8800 ± 1900	26 ± 6.4 (4)
β_h-endorphin	85 ± 8.1	420 ± 60	5.1 ± 0.94 (4)

The values are the means ± s.e.mean; the number of observations is given in parentheses. Tissues were pre-incubated with 100 nM β-funaltrexamine (β-FNA) for 30 min, then washed for 60 min with drug-free Krebs solution. Dose-ratio is the ratio of IC_{50} after pre-incubation with β-FNA to IC_{50} before treatment.

Table 4 Dissociation constants and binding capacities of [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin and of [^{3}H]-(−)-bremazocine in homogenates of the myenteric plexus-longitudinal muscle preparation of the guinea-pig at 25°C

	Multiple binding sites	*Single binding site*	
	Binding capacity	*Dissociation constant*	*Binding capacity*
Ligand	(pmol g^{-1} *tissue*)	(K_D, nM)	(pmol g^{-1} *tissue*)
[^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin	—	2.27 ± 0.28	0.41 ± 0.06 (3)
[^{3}H]-(−)-bremazocine	1.74;1.60	0.32;0.52	0.85;0.79

The values are the means ± s.e.mean; the number of observations is given in parentheses. The binding capacities were calculated from saturation curves and the K_D values from Hill plots. Suppression of the μ- and δ-binding of [^{3}H]-(−)-bremazocine was obtained with 250 nM each of unlabelled [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin and [D-Ala^2, D-Leu^5]enkephalin.

ephalin nor [D-Pen^2, D-Pen^5]enkephalin can inhibit the contractions by more than 50%. In addition to this reduction, the slopes of the log dose-response curves are reduced. This indicates that the hamster vas deferens contains few spare δ-receptors. In the rabbit vas deferens, which contains only κ-receptors, after pre-incubation with β-funaltrexamine there was a decrease in the IC_{50} value of U-50,488H, such as often occurs in this preparation with time (Oka *et al.*, 1981).

In the rat vas deferens which has μ-receptors and possibly ε-receptors, pre-incubation with β-funaltrexamine increased the IC_{50} value of the selective μ-agonist [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin and of β_h-endorphin (Table 3).

Binding characteristics of tritiated opioids in homogenates of the guinea-pig myenteric plexus

In the guinea-pig myenteric plexus the maximum binding capacity for opioids was 1.67 pmol g^{-1} determined with [^{3}H]-(−)-bremazocine which binds to μ-, δ- and κ-sites (Table 4). When binding of [^{3}H]-(−)-bremazocine to the μ- and δ-sites was suppressed, the binding capacity at the κ-sites was 0.82 pmol g^{-1} and the K_D was 0.42 nM. The maximum binding capacity of the selective [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin was 0.41 pmol g^{-1} and the K_D was 2.27 nM. Thus, of the total number of opioid binding sites, 49% are κ-sites, 25% are μ-sites and the remaining 26% are probably δ-sites. The proportion of the three sites in the guinea-pig myenteric plexus is similar to that found in guinea-pig brain where 44% are κ-sites, 24% are μ-sites and 32% are δ-sites. However, the affinity of [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin for the μ-site and of bremazocine for the κ-sites are 2.4 and 6.5 times lower in the myenteric plexus than they are in the brain (Robson *et al.*, 1985).

Interaction of β-funaltrexamine with opioid binding sites

In homogenates of guinea-pig brain, β-funaltrexamine inhibited binding at the μ-, δ- and κ-sites with K_i values of 0.40 nM, 17.9 nM and 2.75 nM, respectively (Table 5). In the myenteric plexus, β-funaltrexamine was also a more potent inhibitor of binding at the μ-site (K_i = 0.83 nM) than at the κ-site (K_i = 12.1 nM) but the K_i values at both sites are higher than those in the brain.

Table 5 The inhibitory effects of β-funaltrexamine on the binding at μ-, δ- and κ-sites in homogenates of the brain and the myenteric plexus-longitudinal muscle preparation of the guinea-pig at 25°C

	μ-site	*δ-site*	*κ-site*
Tissue	K_i (nM)	K_i (nM)	K_i (nM)
Brain	0.401 ± 0.032 (4)	17.9 ± 2.5 (3)	2.75 ± 0.46 (3)
Myenteric plexus	0.830 ± 0.158 (3)	—	12.1 ± 3.0 (3)

The values are the means ± s.e.mean; the number of observations is given in parentheses. In brain homogenates, the μ-site was labelled with [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin (1 nM), the δ-site with [^{3}H]-[D-Ala^2, D-Leu^5]enkephalin (0.7 nM) in the presence of 30 nM unlabelled [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin to suppress μ-binding and the κ-site with [^{3}H]-(−)-bremazocine (0.1 nM) in the presence of 100 nM each of unlabelled μ-and δ-ligands. In homogenates of myenteric plexus-longitudinal muscle, the μ-site was labelled with [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin (6 nM) and the κ-site with [^{3}H]-(−)-bremazocine (0.5 nM) in the presence of 250 nM each of unlabelled μ- and δ-ligands. The Hill coefficients varied from 0.83 to 1.08.

Effects of pre-incubation with β-funaltrexamine on the binding at μ-and κ-sites in homogenates of guinea-pig myenteric plexus

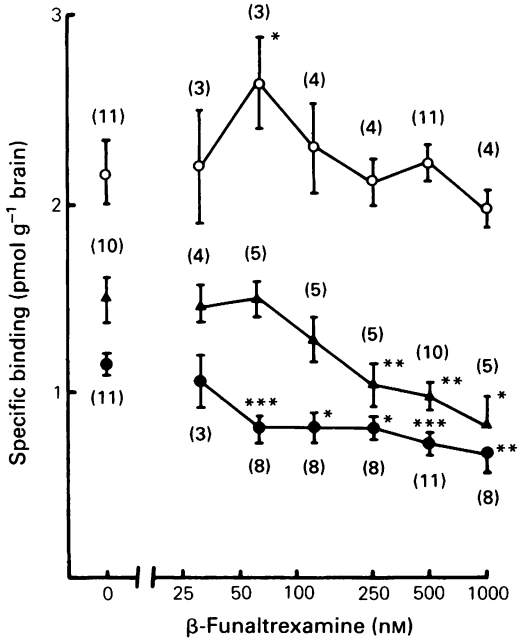
Pre-incubation of homogenates of myenteric plexus with 100 nM or 1000 nM β-funaltrexamine for 30 min did not alter the μ-binding of 6 nM [^{3}H]-[D-Ala^2,

Table 6 The effects of pre-incubation with β-funaltrexamine (β-FNA) on the binding of [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5] enkephalin and of [^{3}H]-(-)-bremazocine in homogenates of the myenteric plexus-longitudinal muscle preparation of the guinea-pig at 25°C

	Binding (pmol g^{-1} *tissue)*		
	[^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5] enkephalin	*[^{3}H]-(−)-bremazocine*	*[^{3}H]-(−)-bremazocine (μ- + δ-binding suppressed)*
Pre-incubation	*μ-site*	*μ+δ+κ-sites*	*κ-site*
(a) 30 min			
No β-FNA	0.32 ± 0.03	0.79 ± 0.03	0.58 ± 0.02
β-FNA 100 nM	0.31 ± 0.05	0.86 ± 0.05	0.61 ± 0.02
β-FNA 1000 nM	0.33 ± 0.06	0.81 ± 0.01	0.60 ± 0.01
(b) 60 min			
No β-FNA	0.44 ± 0.01	0.52 ± 0.01	0.39 ± 0.04
β-FNA 100 nM	0.48 ± 0.04	0.60 ± 0.02	0.44 ± 0.03
β-FNA 1000 nM	0.46 ± 0.06	0.59 ± 0.04	0.46 ± 0.05

The values are the means ± s.e. mean of 3 or 4 observations. The μ-site was labelled with [^{3}H]-[D-Ala^2,MePhe^4, Gly-ol^5] enkephalin (6 nM) and the binding of [^{3}H]-(−)-bremazocine (0.5 nM) was determined with and without suppression of μ- and δ-binding with 250 nM each of unlabelled [D-Ala^2,MePhe^4,Gly-ol^5] enkephalin and [D-Ala^2, D-Leu^5] enkephalin. Homogenates were incubated for 30 or 60 min, at 37°C, in the presence or absence of β-funaltrexamine and then washed twice with drug-free Tris buffer as described in the Methods.

MePhe^4, Gly-ol^5]enkephalin or the κ-binding of 0.5 nM [^{3}H]-(−)-bremazocine (Table 6). If μ-binding and δ-binding are not suppressed, [^{3}H]-(−)-bremazocine binds to μ- and δ-sites in addition to κ-sites; this binding of [^{3}H]-(−)-bremazocine was not affected by pre-incubation with β-funaltrexamine. When the pre-incubation with β-funaltrexamine was extended to 60 min, the binding of both tritiated ligands remained unaffected.



Effects of pre-incubation with β-funaltrexamine on opioid binding in homogenates of guinea-pig brain

Pre-incubation of homogenates of guinea-pig brain for 60 min with different concentrations of β-funaltrexamine had varying effects on the binding at the μ-, δ- and κ-sites (Figure 1). The binding at the μ-site was significantly reduced after pre-incubation with concentrations of β-funaltrexamine greater than 250 nM, whereas at the δ-site binding was decreased with concentrations of β-funaltrexamine greater than 62.5 nM. At the κ-site, binding was increased after pre-incubation with 62.5 nM β-funaltrexamine but was unaffected at other concentrations of up to 1000 nM.

When the homogenate was pre-incubated with 500 nM β-funaltrexamine there was no change in κ-binding after 120 min. In contrast, μ-binding was reduced when the time of incubation was 60 min or longer and δ-binding was decreased when the time of incubation was longer than 30 min (Figure 2).

Figure 1 The effect of pre-incubation for 60 min with different concentrations of β-funaltrexamine on binding at the (▲) μ-, (●) δ- and (○) κ-binding sites in homogenates of guinea-pig brain at 25°C. The μ-site was labelled with [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5] enkephalin (1 nM), the δ-site with [^{3}H]-[D-Ala^2,D-Leu^5] enkephalin (0.7 nM) in the presence of 30 nM unlabelled [D-Ala^2,MePhe^4,Gly-ol^5] enkephalin and the κ-site with [^{3}H]-(−)-bremazocine (0.1 nM) in the presence of 100 nM each of unlabelled [D-Ala^2,MePhe^4,Gly-ol^5] enkephalin and [D-Ala^2,D-Leu^5] enkephalin. Each point represents the mean of (n) experiments as indicated; vertical lines represent s.e. mean. *$P<0.05$; **,$P<0.01$; ***,$P<0.001$; calculated by Student's paired t test.

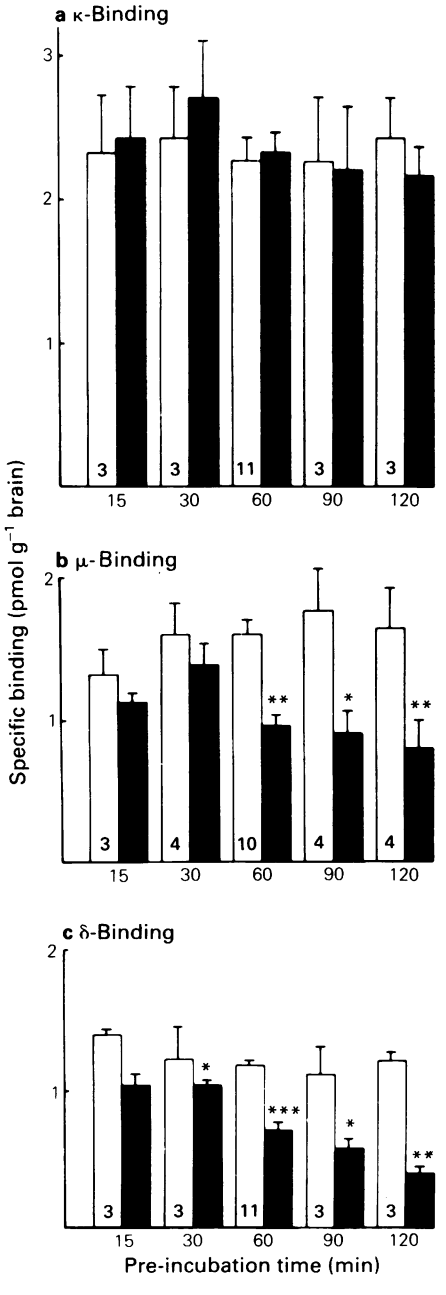


Figure 2 The effects of different times of pre-incubation, in the absence (open column) and presence (solid column) of 500 nM β-funaltrexamine on the binding at (a) κ-, (b) μ- and (c) δ-sites in homogenates of guinea-pig brain at 25°C. Other details are as in Figure 1. Each column represents the mean of (*n*) experiments as indicated; vertical lines represent s.e. mean. $*P<0.05$; $**P<0.01$; $***P<0.001$; calculated by Student's paired *t* test.

Discussion

In an analysis of the pharmacological actions of β-funaltrexamine it has been necessary to distinguish between its acute effects and the effects obtained after prolonged exposure to high concentrations, for which the term pre-incubation is used. In acute experiments, on the guinea-pig myenteric plexus-longitudinal muscle, its main agonist action is on κ-receptors. This view is based on the fact that naloxone has a high K_e value against the agonist actions of β-funaltrexamine; furthermore, its agonist potency is not changed when the μ-receptors have been blocked by prolonged exposure to high concentrations of β-funaltrexamine followed by thorough washing to remove excess of the compound (Takemori *et al.*, 1981; Ward *et al.*, 1982b). In a subsequent investigation, it was shown that antagonists but not agonists could prevent the effects of pre-incubation with β-funaltrexamine in the myenteric plexus. From these observations it has been proposed that β-funaltrexamine and naloxone may exert their effects by interacting with a regulatory site that is allosterically coupled to the μ-binding site (Portoghese & Takemori, 1983).

The findings in this paper support the view that in the guinea-pig myenteric plexus β-funaltrexamine can interact with κ-receptors because it is an agonist in the rabbit vas deferens which has only κ-receptors and is inactive in the hamster vas deferens which has recently been shown to have δ-receptors but no μ- or κ-receptors (McKnight *et al.*, 1984a). As will be discussed in the following paragraphs, pre-incubation of the myenteric plexus with β-funaltrexamine decreases the potency of the selective μ-agonist [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin.

The results obtained with competitive displacement assays indicate that β-funaltrexamine has binding affinities at μ-, δ- and κ-sites. In homogenates of the guinea-pig myenteric plexus as of the brain, β-funaltrexamine is a potent inhibitor of the binding of the highly selective μ-ligand, [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin but is a less potent inhibitor of the binding of [^{3}H]-(−)-bremazocine to κ-sites. This apparent discrepancy between bioassays and binding assays is of particular interest and will be considered later.

For a satisfactory analysis of the effects of pre-incubation with β-funaltrexamine on the opioid receptors in various tissues it is necessary to use highly selective ligands. In bioassays in the myenteric plexus, pre-incubation with high concentrations of β-funaltrexamine increases the IC_{50} value of the selective μ-ligand, [D-Ala^2, MePhe^4, Gly-ol^5]enkaphalin but does not affect the IC_{50} value of the selective κ-ligand, U-50,488H. Notwithstanding a 60-fold increase in the IC_{50} value of [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin, the K_e value of naloxone against the agonist activity of this

μ-ligand is not significantly increased. Only a pre-incubation with 100 nM β-funaltrexamine for 90 min or 1000 nM β-funaltrexamine for 30 min causes a minor rise in the K_e value to 3.1 or 4.7 nM. These values are considerably lower than the K_e value of naloxone for antagonism at κ-receptors (> 25 nM; see Paterson *et al.*, 1984). Therefore, the maximum concentrations of β-funaltrexamine tolerated by the myenteric plexus do not block the activity of all μ-receptors. The remaining receptors are sufficient to give a complete inhibition of the electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation.

As would be expected, after pre-incubation with β-funaltrexamine the K_e value of naloxone against the less selective μ-agonist morphine is higher (9 nM; Ward *et al.*, 1982b) than that against the highly selective μ-ligand [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin (about 2 nM).

However, the effects of pre-incubation with β-funaltrexamine are not restricted to agonists interacting with μ-receptors. In the hamster which has only δ-receptors, pre-incubation with β-funaltrexamine increased the IC_{50} value of the highly selective δ-ligand, [D-Pen^2, D-Pen^5]enkephalin. A similar effect is found in the mouse vas deferens whereas in the rabbit vas deferens the IC_{50} value of the κ-selective agonist U-50,488H is not affected.

For an interpretation of the blocking effects of β-funaltrexamine on the activity of μ-receptors in the guinea-pig myenteric plexus, it is imporant to correlate the findings in the bioassays with the results obtained from binding studies in homogenates of the myenteric plexus. It is striking that, in homogenates, there is no change in the binding of the selective μ-ligand [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin after exposure to β-funaltrexamine for 60 min with concentrations as high as 1000 nM whereas in the myenteric plexus-longitudinal muscle preparation the agonist potency of [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin is much reduced after an exposure to 100 nM β-funaltrexamine for 30 min (Tables 2 and 6).

It should be added that the binding at the μ- and δ-sites is more readily affected by pre-incubation of homogenates of brain than of homogenates of myentric plexus (Figures 1 and 2). In contrast, binding at the κ-site is not affected by exposure to high concentrations of β-funaltrexamine for 2 h.

From a consideration of the data presented in this paper the following conclusion may be drawn. The decrease in the potencies of μ-ligands in the myenteric plexus after pre-incubation with β-funaltrexamine is not due to blockade of μ-binding sites but to interference with the coupling between the binding site and the effector system. At present the precise site of action of β-funatrexamine is not known.

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