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μ-Opioid receptor specific antagonist cyprodime: characterization by in vitro radioligand and [^{35}S]GTPγS binding assays

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Abstract

The use of compounds with high selectivity for each opioid receptor (μ, δ and κ) is crucial for understanding the mechanisms of opioid actions. Until recently non-peptide μ-opioid receptor selective antagonists were not available. However, *N*-cyclopropylmethyl-4,14-dimethoxy-morphinan-6-one (cyprodime) has shown a very high selectivity for μ-opioid receptor in in vivo bioassays. This compound also exhibited a higher affinity for μ-opioid receptor than for δ- and κ-opioid receptors in binding assays in brain membranes, although the degree of selectivity was lower than in in vitro bioassays. Cyprodime has recently been radiolabelled with tritium resulting in high specific radioactivity (36.1 Ci/mmol). We found in in vitro binding experiments that this radioligand bound with high affinity (K_d 3.8 ± 0.18 nM) to membranes of rat brain affording a B_{max} of 87.1 ± 4.83 fmol/mg. Competition studies using μ, δ and κ tritiated specific ligands confirmed the selective labelling of cyprodime to a μ-opioid receptor population. The μ-opioid receptor selective agonist [D-Ala^2, *N*-MePhe^4,Gly^5-ol]enkephalin (DAMGO) was readily displaced by cyprodime (K_i values in the low nanomolar range) while the competition for δ- ([D-Pen^2,D-Pen^5]enkephalin (DPDPE)) and κ- (5α,7α,8β-(−)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl]-benzene-acetamide (U69,593)) opioid receptor selective compounds was several orders of magnitude less. We also found that cyprodime inhibits morphine-stimulated [^{35}S]GTPγS binding. The EC_{50} value of morphine increased about 500-fold in the presence of 10 μM cyprodime. These findings clearly indicate that cyprodime is a useful selective antagonist for μ-opioid receptor characterization.

Keywords: Cyprodime; μ-Opioid receptor; Radioligand; GTPγS binding; Morphine

1. Introduction

Opioid drugs and opioid peptides produce their pharmacological effects, including antinociception, by interaction with opioid receptors in the central nervous system. Opioid receptors are known to be a heterogeneous population consisting of at least three major types (μ, δ and κ) which exhibit different ligand selectivity profiles (Borsodi and Tóth, 1995). Most endogenous opioids and synthetic ligands do not possess absolute specificity for a given receptor type, but interact with more than one opioid receptor. The situation is further complicated by the fact that multiple receptor types may coexist within a single tissue, or even a single cell (Borsodi, 1991).

The multiplicity of opioid receptors is generally accepted and the primary structure of the δ-opioid receptor

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(Kieffer et al., 1992; Evans et al., 1993), μ-opioid receptor (Chen et al., 1993) and κ-opioid receptor (Reisine and Bell, 1993) are known. The further development of highly selective ligands remains a challenge for better characterization for each receptor type and possible subtypes.

Opioid receptor antagonists have been indispensable pharmacological tools for identifying receptor types involved in the actions of endogenous and synthetic opioid receptor agonists. Antagonists are especially useful when the pharmacological endpoints are identical (e.g., antinociception or the inhibition of a smooth muscle contractions), and when it is not easy to distinguish among μ-, δ- and κ-opioid receptor mediated effects. Matthes et al. have recently reported that the μ-opioid receptor gene product is the molecular target of morphine in vivo and that it is a mandatory component for the main pharmacological responses of this opioid (Matthes et al., 1996).

It is known that opioid receptors exert their biological functions by interacting with GTP binding proteins. G_i/G_o proteins to which the opioid receptors are coupled regulate effector molecules such as adenylyl cyclase and/or ion channels (Standifer and Pasternak, 1997). Signal transduction can be monitored in membrane preparations by measuring the binding of the non-hydrolysable GTP analogue, guanosine-5′-*O*-(γ-thio)triphosphate (GTPγS) as a function of the amount of a given ligand (Traynor and Nahorski, 1995).

Cyprodime (*N*-cyclopropylmethyl-4,14-dimethoxymorphinan-6-one) has been shown to be a selective μ-opioid receptor antagonist by using guinea pig ileal longitudinal muscle preparations, rat and mouse vas deferentia and acetic-acid writhing tests (Schmidhammer et al., 1989).

In the present study, we have further defined the in vitro ligand-binding profile of cyprodime and described the biochemical characterization of its tritiated derivative, [^{3}H]cyprodime. We have also evaluated the functional effectiveness of cyprodime to alter [^{35}S]GTPγS binding and to inhibit morphine-stimulated [^{35}S]GTPγS binding.

2. Materials and methods

2.1. Chemicals

Cyprodime was synthesized as previously reported (Schmidhammer et al., 1989). D-Phe-Cys-Tyr-D-Arg-Thr-Pen-Thr-NH_2 (CTAP) and [D-Ser^2, Leu^5, Thr^6]enkephalin (DSLET) were a generous gift from the National Institute of Drug Abuse Drug Supply System (Rockville, MD). (±)Ethylketocyclazocine methanesulfonate was supplied by Sterling Winthrop Research Institute (Rennsealeer, New York). Dihydromorphine, deltorphin II, Tyr-Tic-Phe-Phe-OH (TIPP) and Ile^{5,6}deltorphin II were synthesized as previously reported (Tóth et al., 1982a; Buzas et al., 1992; Nevin et al., 1993, 1994, respectively). 5α,7α,8β-(−)-*N*-Methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl]-benzene-acetamide (U69,593) and *trans*-3,4-dichloro-*N*-methyl-1-*N*-1-pyrrolidinyl-(cyclohexyl)-benznecetamide (U50,488) were obtained from Upjohn (Kalamazoo, MI). [D-Ala^2, *N*-MePhe^4,Gly^5-ol]enkephalin (DAMGO), was purchased from Bachem (Switzerland). All other chemicals were of analytical grade.

[^{3}H]Cyprodime (31.6 Ci/mmol) (Ötvös et al., 1992) and [^{3}H]naloxone (72 Ci/mmol) (Tóth et al., 1982b) were synthesized in our Isotope Laboratory as previously reported. [^{3}H]U69,593 (43 Ci/mmol) was purchased from DuPont-New England Nuclear (Boston, MA, USA) and [^{3}H]DAMGO (59 Ci/mmol) and [^{3}H][D-Pen^2,D-Pen^5]enkephalin (DPDPE) 32 Ci/mmol) from Amersham (Buckinghamshire, England).

2.2. Membrane preparation

Rat brain membranes were prepared according to Pasternak et al. (1975) with a small modification. Rats (PVG/C and Wistar strain) were decapitated. The brains without cerebellum were removed and then homogenized in twenty volumes (w/vol) of ice-cold Tris–HC1 buffer (50 mM, pH 7.4) and centrifuged (40,000 × *g*, 4°C, 20 min). The final pellet was resuspended in buffer (50 mM Tris–HC1, pH 7.4) and incubated for 30 min at 37°C. Centrifugation was repeated and the final pellet resuspended in buffer (50 mM Tris–HC1, 0.32 M sucrose, pH 7.4) and stored at −70°C. The guinea pig brain membranes were prepared similarly.

2.3. Radioligand binding assays

Ligand binding experiments were carried out in 50 mM Tris–HC1 buffer (pH 7.4) with or without 100 mM NaCl, in a final volume of 1 ml containing approximately 0.3–0.5 mg protein. Incubations were started by addition of membrane suspension and continued in a shaking water bath until steady-state was achieved (40–45 min). The reaction was terminated by rapid filtration on a Brandel M24R cell harvester through Whatmann GF/B or GF/C filters and washed with 3 × 5 ml of ice-cold buffer. The filters were dried and the bound radioactivity was determined in a toluene based scintillation cocktail in Wallac 1409 liquid scintillation counter. Total binding was defined as that measured in the absence of competing agent and non-specific binding as binding remaining in the presence of 10 μM naloxone.

2.4. [^{35}S]GTPγS binding

For [^{35}S]GTPγS binding the same membrane preparation was used. Tubes contained 10 μg of protein, 30 μM GDP, 10^{-10} to 10^{-5} M opioid receptor ligands, and 0.05 nM [^{35}S]GTPγS, all in 50 mM Tris–HCl buffer containing 1 mM EGTA and 3 mM $MgCl_2$ in a final volume of 1 ml. Tubes were incubated for 1 h at 30°C. Total activity was

measured in the absence of tested compounds, and nonspecific binding was measured in the presence of 100 μM non-labelled GTPγS. The incubation was terminated by filtrating the samples through Whatman GF/B glass fiber filters. Filters were washed three times with ice-cold buffer in a Millipore filtration instrument, then dried. Radioactivity was measured in a Wallac 1409 scintillation counter using a toluene based scintillation cocktail. Stimulation is given as percentage of specific binding. Data were calculated from three or four separate experiments done in triplicate.

2.5. Data analysis

All assays were carried out at least three times in duplicate, and values are given as means ± S.E.M. The binding capacity (B_{max}) and K_d of [^{3}H]cyprodime were calculated according to Rosenthal (1967) using GraphPad Prism 2.01 computer program. Competition data were analyzed with the program LIGAND (Munson and Rodbard, 1980), using a non-linear least squares fitting algorithm.

3. Results

3.1. Competition assays

The selectivity of unlabelled cyprodime was tested in rat brain membranes using highly selective radioligands for each receptor (μ, δ and κ) (Table 1). The μ-opioid receptor selective peptide, [^{3}H]DAMGO, was readily displaced by cyprodime (K_i value 5.4 nM). Cyprodime showed much less affinity for δ binding sites, which were labelled with [^{3}H]DPDPE. More than 40-fold difference was observed (244.6 for DPDPE vs. 5.4 nM for DAMGO K_i values) when compared with binding to [^{3}H]DAMGO. Almost a similar low affinity (K_i 213.7 nM) was found when cyprodime competed for the κ binding sites labelled with [^{3}H]U69,593.

Further characteristics of cyprodime in rat brain were investigated when [^{3}H]naloxone, a general opioid receptor antagonist, was displaced in the absence and presence of 100 nM NaCl. Without the salt, a K_i value of 14.95 ± 1.01 nM was estimated, which did not change upon addition of NaCl (K_i value 14.70 ± 1.92 nM). This result clearly demonstrates the antagonistic property of cyprodime.

Table 1
Affinity of cyprodime for opioid receptors labelled with different tritiated ligands
Rat brain membranes were incubated with [^{3}H]DAMGO for 45 min at 35°C, [^{3}H]DPDPE for 150 min at 25°C and [^{3}H]U69,593 for 30 min at 30°C with 11 concentrations of cyprodime (10^{-5} to 10^{-12}). Values represent means ± S.E.M. from three separate experiments.

Tritiated ligands	Specificity	K_i (nM)
[^{3}H]DAMGO	μ	5.4 ± 2.4
[^{3}H]DPDPE	δ	244.6 ± 23.1
[^{3}H]U69,593	κ	2187 ± 42.3

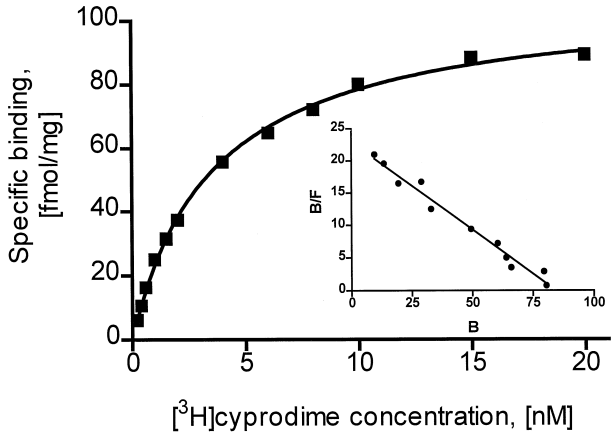

Fig. 1. Equilibrium saturation binding isotherm for [^{3}H]cyprodime binding to rat brain (Wistar strain). Membranes were incubated for 40 min at 25°C. Specific binding was measured at 12 concentrations of radioligand varying from 0.1 to 20 nM using 10 mM naloxone to define nonspecific binding. The insert represents the Rosenthal transformation of the equilibrium binding isotherm. Prism 2.01 computer program was used to fit experimental data and the following parameters were calculated: K_d, 3.8 nM and B_{max}, 87.1 fmol/mg protein.

3.2. Binding of [^{3}H]cyprodime

Binding of [^{3}H]cyprodime to rat brain membranes reached equilibrium at 25°C in 40 min and was stable for at least 90 min. In the saturation experiments, a single class of binding site was detected with a K_d value of 3.83 ± 0.18 nM. The binding capacity was found to be 87.1 ± 4.83 fmol/mg protein in Wistar rats (Fig. 1). The same affinity (3.84 ± 0.12 nM) was observed when another strain (PVG/C) was used. However, it is interesting to note that the B_{max} value was significantly ($p < 0.01$) higher (124 ± 13 fmol/mg protein) in this strain.

Specifically bound [^{3}H]cyprodime was displaced readily from rat brain by cold cyprodime (K_i 8.1 nM) and by the μ-opioid receptor agonists dihydromorphine (K_i 0.4 nM) and DAMGO (K_i 1.1 nM) (Table 2). The μ-opioid receptor selective somatostatin analogue, CTAP, showed less affinity (K_i 43.8 nM). On the other hand, the mixed opioid receptor antagonist, naloxone, exhibited high affinity (K_i 0.9 nM). The weak labelling of δ- and κ-opioid receptors was confirmed by the low affinities of the δ- (deltorphin II and Ile^{5,6}deltorphin II) and κ- (norbinaltorphimine and U50,488) opioid receptor selective ligands. Of the highly δ-opioid receptor specific agonists, deltorphin II and Ile^{5,6}deltorphin II showed very low affinity K_i values, 1186 nM and 1900 nM, respectively. The δ-opioid receptor specific antagonist TIPP was even less potent (K_i 2827 nM). DSLET, the fairly δ-opioid receptor selective ligand which shows cross reactivity with μ-opioid receptor (Gacel

Table 2
Displacement of [^{3}H]cyprodime by opioid ligands in membranes of rat and guinea pig brain
Membranes were incubated with 2 nM [^{3}H]cyprodime for 40 min at 25°C with 11 concentrations (10^{-5} to 10^{-12}) of each competing ligand. Values represent mean ± S.E.M. from three observations. N.T. means not tested.

Competing ligands		K_i (nM)	
		Rat brain (Wistar)	Guinea pig brain
Cyprodime	μ-opioid receptor antagonist	8.1 ± 1.8	26.6 ± 3.9
Dihydromorphine	μ-opioid receptor agonist	0.4 ± 0.1	6.0 ± 2.3
DAMGO	μ-opioid receptor agonist	1.1 ± 2.3	2.6 ± 1.9
CTAP	μ-opioid receptor antagonist	43.8 ± 33.0	48.1 ± 26.9
Naloxone	mixed opioid receptor antagonist	0.9 ± 0.03	1.4 ± 0.5
DSLET	δ-opioid receptor agonist	9.8 ± 2.4	6.3 ± 3.7
TIPP	δ-opioid receptor antagonist	2827 ± 1243	7060 ± 850
Deltorphin II	δ-opioid receptor agonist	1186 ± 104	2878 ± 1242
Ile^{5,6}-deltorphin II	δ-opioid receptor agonist	1900 ± 98	N.T.
U-50,488	κ-opioid receptor agonist	288.1 ± 5.5	303.7 ± 112.8
Norbinaltorphimine	κ-opioid receptor antagonist	171.6 ± 57.0	769.8 ± 321.8

et al., 1988) competes with relatively high affinity (K_i 9.8 nM) for [^{3}H]cyprodime. The affinity of the highly selective κ-opioid receptor agonist U50,488 is much lower (K_i 288.1 nM) and the κ-opioid receptor selective antagonist norbinaltorphimine showed a comparable low affinity (K_i 171.6 nM).

In guinea pig brain, all ligands except DSLET, showed higher K_i values than in rat brain. Cyprodime itself had about 3× less affinity (K_i 26.6 nM), and DAMGO showed about 2.5× less affinity (K_i 2.6 nM) than in rat brain. Naloxone still exhibited high affinity (K_i 1.4 nM) whereas dihydromorphine showed a somewhat decreased affinity (K_i 6.0 nM). DSLET exhibited about the same affinity in guinea pig (K_i 6.3 nM) as in rat brain (K_i 9.8 nM). The δ-opioid receptor selective agonist deltorphin II showed a K_i value in the micromolar range (K_i 2878 nM) while the δ-opioid receptor antagonist peptide TIPP was even less potent (K_i 7060 nM). The κ-opioid receptor specific ligands displayed higher affinities than the δ-opioid receptor ligands where K_i values for U50,488 and norbinaltophimine were found to be 303.7 nM and 769.8 nM, respectively.

3.3. [^{35}S]GTPγS binding

We first examined the effects of increasing concentrations of cyprodime on [^{35}S]GTPγS binding. Morphine, a potent μ-opioid receptor agonist, was used as a reference compound. The responses in this assay were detected in the concentration range from 10^{-9} to 10^{-5} M (Fig. 2). Cyprodime caused a slight, but not significant increase of the amount of bound [^{35}S]GTPγS. Thus, the maximal stimulation induced by cyprodime was about 110% above the basal value, while morphine reached a plateau at 155%.

In further experiments, the effects of two different concentrations of cyprodime were studied (Fig. 3). Morphine was incubated either with buffer (negative control), 1 μM cyprodime, 10 μM cyprodime or 1 μM naloxone (positive control). When morphine was incubated with buffer alone an EC_{50} of 244 nM was detected with 155%

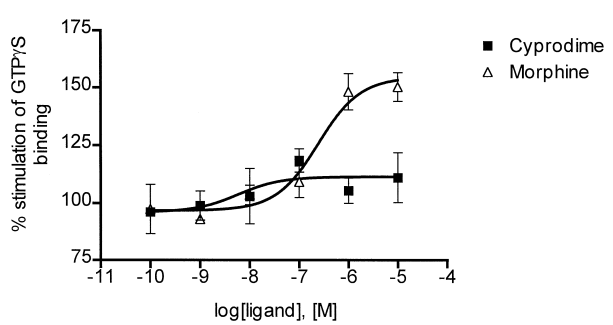

Fig. 2. Effect of different concentrations of morphine [△] and cyprodime [■] on [^{35}S]GTPγS binding to G proteins in crude rat brain membrane preparations. Total binding [without any stimulating agent] is 100%. Data points achieved by the addition of cyprodime do not differ significantly from the basal line. Assay tubes contained 10 mg of protein, 0.05 nmol [^{35}S]GTPγS, 30 mmol GDP, 1 mM EGTA and 3 mM $MgCl_2$ in Tris–HCl buffer, pH 7.4. Incubation was carried out for 60 min at 30°C. Experiments were done three times in triplicate. Data are mean ± S.E.M.

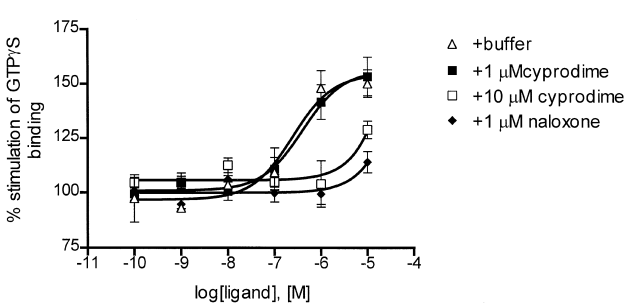

Fig. 3. Stimulation of [^{35}S]GTPγS binding to crude rat brain membranes by various concentrations of morphine was tested in the presence of 0 [△], 1 [■] and 10 [□] mM cyprodime or 1 mM [◆] naloxone. Stimulation of [^{35}S]GTPγS (50 pmol) binding to crude rat brain membranes (10 mg/tube). Assays were performed in the presence of 30 mM GDP for 60 min at 30°C. Points represent means ± S.E.M. from three separate experiments done in triplicate.

maximal stimulation, in agreement with the results of the previous experiment. 1 μM naloxone abolished the stimulating effects of morphine almost completely (EC_{50} ~ 100 μM). 1 μM cyprodime had no significant effect on the morphine dose–response curve when incubated at the lower concentration (1 μg). However, a higher dose of cyprodime (10 μg) dramatically reduced the stimulatory responses of morphine on GTPγS binding. At this concentration of cyprodime, the dose–response curve of morphine shifted to the right, revealing a 500-fold increase of the EC_{50}.

4. Discussion

The present findings with [^{3}H]cyprodime strongly support previous bioassay data which indicated cyprodime to be a highly selective μ-opioid receptor antagonist (Schmidhammer et al., 1989). The μ/κ selectivity ratio in the guinea pig ileum was found to be 37, while in the isolated mouse vas deferens preparation it was 28, which were 2 to 3× greater values than with naloxone. In the mouse vas deferens preparation the μ/δ selectivity ratio was 15× greater than with naloxone (Schmidhammer et al., 1989). In the present radioligand binding assays highly selective compounds [^{3}H]DAMGO, [^{3}H]DPDPE and [^{3}H]U69,593 were used in rat brain membranes for labelling μ-, δ- and κ-opioid receptors, respectively. Unlabelled cyprodime displaced [^{3}H]DAMGO with high affinity (K_i 5.4 nM). The affinity of cyprodime for the δ and κ sites was more than 40× less. It is interesting to note that the same rank order of affinity was seen in guinea pig and frog brain (data not shown) as in rat. The antagonist property of cyprodime was shown in binding assays where [^{3}H]naloxone was displaced by cyprodime (almost identical K_i values were observed when the experiments were performed in the presence or absence of 100 nM NaCl).

Cyprodime was labelled with tritium resulting in a specific radioactivity of 31.6 Ci/mol (Ötvös et al.,1992). The detailed binding properties of this ligand were investigated. The binding was saturable and a single binding site was detected with high affinity: K_d value of 3.8 nM in two different strains of rats (PVG/C and Wistar). The maximal number of binding sites were 87 and 124 fmol/mg protein in Wistar and PVG/C strains, respectively. Although cyprodime labels a single population of receptors in rat brain, the density is less than that measured by [^{3}H]DAMGO (B_{max} 222 ± 5 fmol/mg) and may indicate that cyprodime is only labelling a subset of μ-opioid receptor, possibly supporting the concept of μ-opioid receptor heterogeneity (Varga et al., 1987).

The selectivity of tritiated cyprodime was tested in rat and guinea pig brain. Unlabelled cyprodime, the μ-opioid receptor agonists dihydromorphine and DAMGO, as well as naloxone showed affinities in the low nanomolar range (K_i 0.4–8.1 nM). Somewhat lower potency was detected with CTAP (K_i 43.8 nM) which is a peptide derivative analogue of somatostatin (Pelton et al., 1986). In guinea pig brain all of the above unlabelled ligands showed affinities in the nanomolar range with somewhat higher K_i values (2.6–26.6 nM). These differences might be related to the different ratio of μ- and κ-opioid receptors in the two species (Benyhe et al., 1992). A number of δ-opioid receptor selective ligands (including DSLET, TIPP, deltorphin II and Ile5,6 deltorphin II) were applied to compete for tritiated cyprodime. Low affinities were observed (K_i values in the micromolar range) when using these compounds in rat as well as guinea pig brain. These findings confirm the high selectivity of tritiated cyprodime. Low affinities were also measured using the κ-opioid receptor selective agonist U50,488 (K_i 288 and 303 nM) and the κ-opioid receptor selective antagonist norbinaltorphimine (K_i 171 and 769 nM) for representing κ-opioid receptor specific compounds.

The most currently used non-peptide antagonists, naloxone and naltrexone, do not exhibit high selectivity for any of the opioid receptors. β-funaltrexamine (Takemori et al., 1986) and CTAP (Pelton et al., 1986) were found to be μ-opioid receptor selective antagonists. However, β-funaltrexamine is a non-competitive ligand for μ-opioid receptor and CTAP which does not cross the blood–brain barrier also has high affinity for somatostatin receptors. Such inconveniences limit the application of these compounds. The basic pharmacological properties of cyprodime have been previously described (Schmidhammer et al., 1989). The selective antagonistic properties of this ligand were shown in the guinea pig ileal longitudinal muscle, mouse vas deferentia and rat vas deferentia preparations. High selectivity ratios for cyprodime were shown in these assays (μ/δ: 74 in rat vas deferentia and 100 in mouse vas deferentia; 6 μ/κ: 28 in mouse vas deferentia and 37 in guinea pig ileal longitudinal muscle preparation).

When used as a tritiated ligand, cyprodime labels a population of receptors in rat brain confirmed to be μ-opioid receptor by competition assay. The high selectivity for μ-opioid receptor found with [^{3}H]cyprodime in the binding study is supported by the high selectivity of cold cyprodime also reported in rat brain membranes in the present paper. These results extend the previous findings (Schmidhammer et al., 1989) suggesting that cyprodime can be a useful pharmacological tool to characterize the μ-opioid receptor.

Besides in vitro binding experiments, functional assays were also performed. The effects of cyprodime on agonist stimulated [^{35}S]GTPγS binding in crude rat brain membrane preparations were studied. These functional experiments confirm the finding that cyprodime is an antagonist at μ-opioid binding sites, since it reversed the stimulatory effects of morphine in the [^{35}S]GTPγS binding assay at a concentration of 10 μM. Inhibition could not be detected at lower concentrations of cyprodime showing a weaker antagonist property in comparison with naloxone, which is in agreement with the previous findings in in vivo phar-

macological assays. Indeed, cyprodime exhibited about one-tenth the potency of naloxone to antagonise morphine-induced antinociception in the acetic acid writhing test in mice, and a similar one-tenth ratio was obtained to modify respiratory activity parameters in rabbits and to precipitate withdrawal syndrome in morphine-dependent mice (Schmidhammer et al., 1989). Cyprodime itself produced a negligible and non-significant stimulatory response on [^{35}S]GTPγS binding, which may reflect a very small degree of agonist property of the compound. In binding assays, cyprodime exhibited a lower potency than naltrexone or naloxone to displace [^{3}H]naloxone in the presence of NaCl, but the binding properties of cyprodime were strongly impaired in the absence of NaCl which suggests a pronounced antagonistic activity in this test (Schmidhammer et al., 1989).

In conclusion, the present study provides binding competition and functional results showing the properties of cyprodime as a selective μ-opioid receptor antagonist and indicates that [^{3}H]cyprodime is a selective μ-opioid receptor radioligand with high affinity that has the potential to be a useful tool in probing μ-opioid receptor mechanisms.

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