Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 494 (2004) 121–130

www.elsevier.com/locate/ejphar

Na^+ modulation, inverse agonism, and anorectic potency of 4-phenylpiperidine opioid antagonists

Paul J. Emmerson, Jamie H. McKinzie, Peggy L. Surface, Todd M. Suter, Charles H. Mitch, Michael A. Statnick*

Endocrine Research, Lilly Research Laboratories, Lilly Corporate Center DC0403, Indianapolis IN 46285, USA

Received 2 February 2004; received in revised form 28 April 2004; accepted 30 April 2004

Abstract

Differences in the anorectic activity of morphinan (e.g., naltrexone) and 3,4-dimethyl-4-(3-hydroxyphenyl)piperidine (4PP) opioid receptor antagonists have been described. In an attempt to explain these differences, the influence of Na^+ on opioid binding affinity and functional activity of 4PP antagonists was compared to other opioid antagonists. The binding affinities of neutral antagonists were unaffected by the addition of Na^+, whereas that for the peptide, inverse agonist *N*,*N*-diallyl-Tyr–Aib–Aib–Phe–Leu–OH (ICI174864) was increased. Similarly, the binding affinities of the 4PP antagonist (3*R*,4*R*)-1-((*S*)-3-hydroxy-3-cyclohexylpropyl)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidine (LY255582) and other 4PP antagonists were increased in the presence of Na^+ with the greatest effects at the delta opioid receptor followed by the mu and kappa opioid receptors, respectively. Similar to ICI174864, 4PP antagonists were found to inhibit basal GTPγ[^{35}S] binding at the delta opioid receptor indicating inverse agonist activity. A correlation was observed between the binding affinities in the presence of Na^+, the inverse agonist potency, and the anorectic potency of 4PP antagonists. These data suggest that 4PP antagonists differ from morphinan antagonists in their inverse agonist activity and suggest a relationship between inverse agonism and anorectic activity.

Keywords: Delta opioid receptor; Mu opioid receptor; Kappa opioid receptor; Inverse agonist

1. Introduction

In the central, peripheral, and enteric nervous systems, activation of opioid receptors by the endogenous opioid peptides plays an important role in both behavioral and homeostatic functions including nociception, reward, pituitary hormone release, respiration, food intake, and gastrointestinal motility (Vaccarino and Kastin, 2001). The actions of the endogenous opioid peptides are mediated by a family of G protein-coupled seven transmembrane receptors designated mu (OP_3), kappa (OP_2), and delta (OP_1) (Dhawan et al., 1996). Agonist binding to these receptors leads to the activation of a cascade of G protein mediated signaling events including a reduction of cAMP levels, inhibition of calcium channels, and activation of potassium channels (Grudt and Williams, 1995). Modulation of these effector systems results in both suppression of neuronal excitation and presynaptic inhibition of neurotransmitter release (Miller, 1998), leading to the observed control of behavior and homeostasis.

Following the initial demonstration of opioid ligand binding, Na^+ was shown to decrease the binding of agonists and increase the binding of antagonists (Pert and Snyder, 1974). The delta opioid inverse agonist *N*,*N*-diallyl-Tyr–Aib–Aib–Phe–Leu–OH (ICI174864) (Costa and Herz, 1989) was further shown to have increased binding affinity at the delta opioid receptor in the presence of Na^+, whereas binding of the neutral antagonist naloxone was unaffected by Na^+ (Ishizuka and Oka, 1984; Appelmans et al., 1986, Emmerson et al., 1994). Protection of opioid receptors from sulfhydryl alkylating agents in the presence of Na^+ suggested these

* Corresponding author. Tel.: +1-317-277-1123; fax: +1-317-276-9722.

E-mail address: m.statnick@lilly.com (M.A. Statnick).

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.04.050

effects result from a conformational change in the receptor protein upon Na^+ binding (Simon and Groth, 1975). The observed differences in Na^+ response further suggest that inverse agonist activity may relate to the preference of a ligand for the G protein-uncoupled receptor over the G protein-coupled receptor (De Ligt et al., 2000; Kenakin, 2001) and that Na^+ responsiveness of opioid binding affinity can discriminate neutral antagonists from inverse agonists.

The endogenous opioid system has been shown to be involved in the control of food intake and body weight (Glass et al., 1999). Opioid receptor antagonists including the series of 3,4-dimethyl-4-(3-hydroxyphenyl)piperidines (4PPs) (Zimmerman et al., 1978) have been identified as potent anorectics (Shaw et al., 1990). Administration of 4PP antagonists to rats results in both a decrease in food intake and body weight (Mitch et al., 1993; Statnick et al., 2003). Interestingly, these effects are greater and longer lasting than the morphinan antagonists naloxone, naltrexone, or nalmefene (Shaw et al., 1990; Shaw, 1993; Mitch et al., 1993). 4PP antagonists exhibit similar affinity and selectivity in vitro as the standard opioid receptor antagonists, suggesting that potency alone cannot explain the difference in efficacy of these antagonists. To further examine the unique pharmacology of this class of opioid receptor antagonists, we examined the modulation of 4PP binding by Na^+ and functional activity in an assay of delta opioid inverse agonism. These results were further compared to the previously determined anorectic potency of 4PP antagonists (Mitch et al., 1993). A preliminary report of these findings has been presented previously (McKinzie et al., 2001).

2. Materials and methods

2.1. Opioid ligands

[^{3}H]diprenorphine, [^{3}H]bremazocine, and [^{35}S]GTPγS were obtained from Perkin-Elmer/NEN. Membranes from CHO cells expressing the human mu, kappa, and delta opioid receptors were obtained from Receptor Biology (Belstville, MD). Naltrexone, diprenorphine, nalmefene, Tyr–D-Ala–Gly–(Me)Phe–Gly-ol (DAMGO), and 5a,7a,8b(2)-*N*-methyl-*N*-(7-Cl-pyrrolidinyl)-1-oxaspiro (4,5)dec-8-yl)benzene acetamide (U69593) were from Sigma (St Louis, MO). *N*,*N*-diallyl-Tyr–α-aminoisobutyric acid–α-aminoisobutyric acid–Phe–Leu–OH (ICI174864) and [D-Pen^2,D-Pen^5]enkephalin (DPDPE) were from Bachem (King of Prussia, PA). A previous report (Mitch et al., 1993) has described the synthesis and structure of the 4PP antagonists (Fig. 1) including LY99335 (number 14), *cis*-3-(1,3,4-trimethyl-4-piperidinyl)-phenol hydrochloride; LY243348 (number 41), (3*R*-*cis*)-3-[3,4-dimethyl-1-[3-(2-thienyl)-propyl]-4-piperidinyl]-phenol hydrochloride; LY243578 (number 42), (3*S*-*cis*)-3-[3,4-dimethyl-1-[3-(2-thienyl)-propyl]-4-piperidinyl]-phenol hydrochloride; LY255609 (number 12), (α*S*,3*S*,4*S*)-α-cyclohexyl-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinepropanol; LY255610 (number 10), (α*R*,3*R*,4*R*)-α-cyclohexyl-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinepropanol; LY255582 (number 11), (α*S*,3*R*,4*R*)-α-cyclohexyl-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinepropanol; LY255265 (number 13), (α*R*,3*S*,4*S*)-α-cyclohexyl-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinepropanol; LY264840 (number 37), 3-[(3*R*,4*R*)-1-hexyl-3,4-dimethyl-4-piperi-

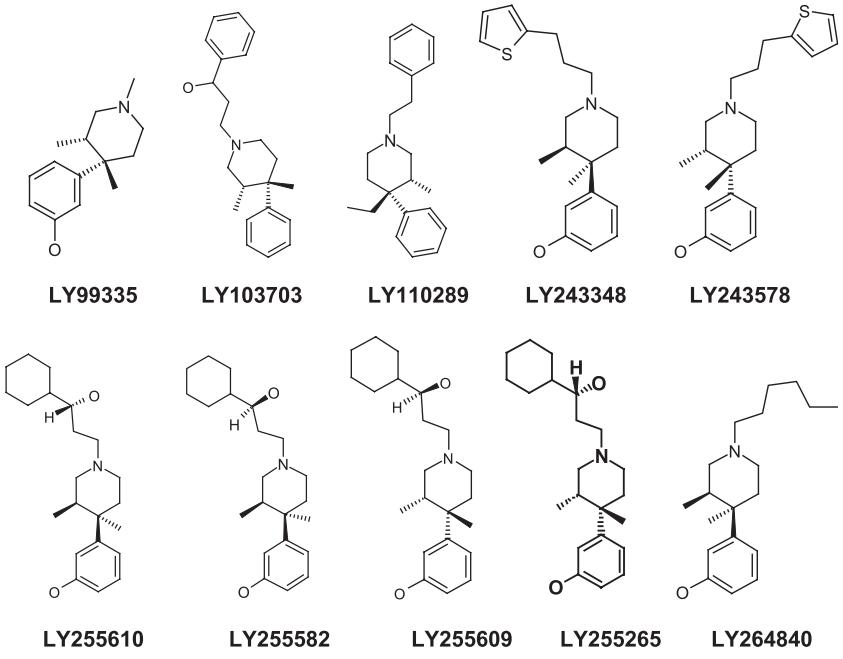

Fig. 1. Structures of 4-phenylpiperidine antagonists.

dinyl]-phenol hydrochloride. The structures of LY103703 and LY110289 are (3*S*,4*S*)-rel-3,4-dimethyl-α,4-diphenyl-1-piperidinepropanol hydrochloride and *cis*-3,4-dimethyl-4-phenyl-1-(2-phenylethyl)-piperidine hydrobromide, respectively (Fig. 1).

2.2. Receptor binding

Radioligand, membrane suspension, and buffer were added to 96-well blocks to yield a 500-μl assay volume. The choice of radioligands ([^{3}H]diprenorphine for mu and kappa opioid receptors and [^{3}H]bremazocine for the delta opioid receptor) was based on the recommendation of commercial suppliers of opioid receptor membranes (Receptor Biology or Biosignal). The buffer contained 50 mM Tris–HCl, 5 mM $MgCl_2$, and 1 mM EDTA (pH 7.4). Membranes were thawed, diluted in buffer, resuspended using a polytron homogenizer, and then added at a concentration of 4 μg (delta opioid receptor) or 6 μg (mu and kappa opioid receptors) per well. Where indicated, NaCl was added to a final concentration of 100 mM. The reaction was incubated for 120 min at room temperature. Samples were filtered through glass fiber filters (Wallac filtermat A) presoaked with 0.05% polyethyleneimine in 50 mM Tris pH 7.4 and washed 3× with 5 ml buffer (50 mM Tris–HCl pH 7.4, 4 °C) using a Tomtec Mach III cell harvester. Filtermats were then dried and imbedded with Meltilex scintillant A and the radioactivity counted in a Wallac Microbeta scintillation counter. Saturation binding was conducted in these membranes in the absence and presence of Na^+ to determine K_d values at the respective receptors (data not shown). A concentration approximating the K_d was used to generate displacement curves. Eleven-point ligand displacement curves were determined using 0.2 nM [^{3}H]diprenorphine (mu and kappa opioid receptors) or 0.5 nM [^{3}H]bremazocine (delta opioid receptor). Specific binding was determined by displacement with 10 μM naltrexone. Curves were plotted as the percent of specific binding, and IC_{50} values were determined using a sigmoidal dose–response curve with variable slope using GraphPad Prism (GraphPad Software, San Diego, CA). K_i values were calculated from the IC_{50} by the equation of Cheng and Prusoff (1973), where $K_i = IC_{50} \times (1 + D \times K_d^{-1})^{-1}$. K_d values determined at the respective receptors in the absence and presence of Na^+ were [^{3}H]diprenorphine, mu (+Na) 0.06 nM, mu (−Na) 0.125 nM, [^{3}H]diprenorphine, kappa (+Na) 0.2 nM, kappa (−Na) 0.2 nM; [^{3}H]bremazocine delta (+Na) 0.5 nM, delta (−Na) 0.5 nM.

2.3. [^{35}S]GTPγS binding assays

[^{35}S]GTPγS binding was determined in a scintillation proximity assay (SPA)-based format (DeLapp et al., 1999). Assays were conducted in a 200-μl volume with the following buffer composition: 100 mM KCl, 20 mM HEPES, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 50 μM GDP, 0.5 nM [^{35}S]GTPγS. Where noted, 100 mM NaCl was substituted for KCl and the concentration of GDP reduced to 3 μM to maintain similar specific GTPγS binding. Delta opioid receptor membrane suspension was added at a concentration of 20 μg protein per well. Wheat germ agglutinin coated SPA beads (Amersham, Arlington Hts., IL) were added at 1 mg per well to detect membrane-bound [^{35}S]GTPγS. The assay contents were added to clear bottom Costar 96-well assay plates in the following volumes: 50 μl ligand, buffer or unlabeled GTPγS, 50 μl of [^{35}S]GTPγS, 50 μl membrane suspension, and 50 μl SPA beads. Plates were sealed and incubated for 2 h at room temperature. Plates were then placed at 4 °C overnight to allow the SPA beads to settle and then counted in a Wallac Microbeta. Specific [^{35}S]GTPγS binding was determined as the difference in CPM observed in the absence and presence of 10 μM unlabeled GTPγS. Data were plotted as the percent of specific [^{35}S]GTPγS bound. EC_{50} and percent change in basal were determined with GraphPad Prism

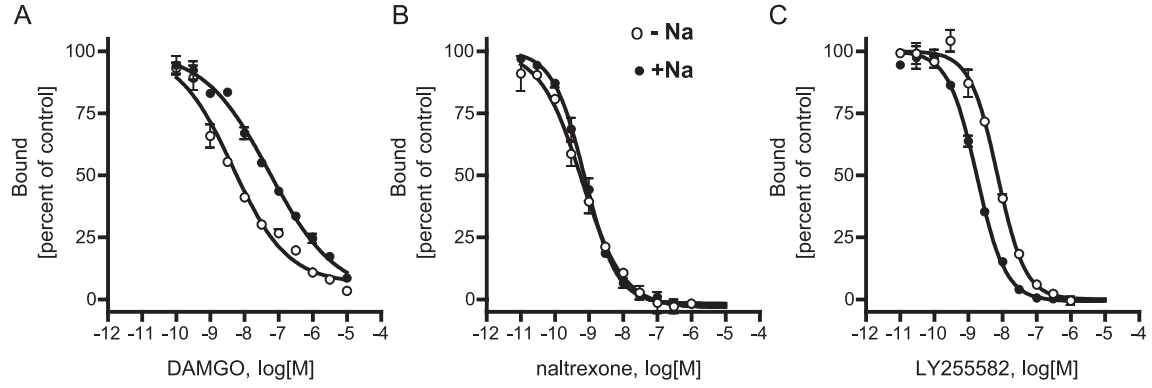

Fig. 2. Sodium effect on DAMGO (A), naltrexone (B), and LY255582 (C) displacement of [^{3}H]diprenorphine from CHO-MOR cell membranes. Data are represented as the percent inhibition of specific binding (0.2 nM) [^{3}H]diprenorphine in the absence (open symbols) and presence (closed symbols) of 100 mM NaCl. Shown are the results of a representative experiment.

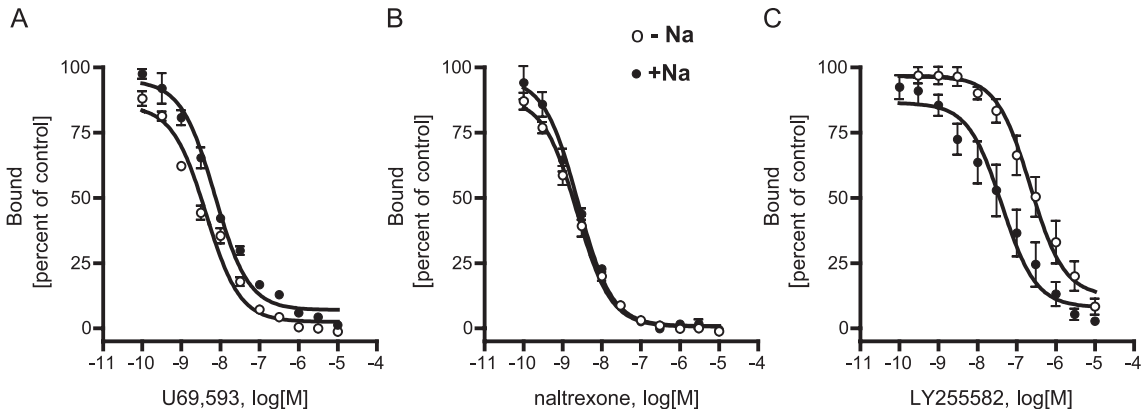

Fig. 3. Sodium effect on U69593 (A), naltrexone (B), and LY255582 (C) displacement of [^{3}H]diprenorphine from CHO-KOR cell membranes. Data are represented as the percent inhibition of specific binding (0.2 nM) [^{3}H]diprenorphine in the absence (open symbols) and presence (closed symbols) of 100 mM NaCl. Shown are the results of a representative experiment.

software using a sigmoidal dose–response curve with variable slope (GraphPad Software).

3. Results

Opioid receptor antagonist binding affinities were determined by the displacement of [^{3}H]diprenorphine or [^{3}H]bremazocine from membranes derived from cells expressing the human mu and kappa or delta opioid receptors, respectively. Ligand-binding affinities were determined in the absence and presence of 100 mM NaCl. In the presence of Na^+, the binding of the mu-selective opioid receptor agonist DAMGO and delta-selective agonist DPDPE were inhibited, while the kappa-selective agonist U69593 was unaffected by Na^+ (Figs. 2–4, Table 1–3). The binding affinities of the opioid receptor antagonists naltrexone, nalmefene, and naltriben were unaffected by Na^+ addition at all three receptors (Figs. 2–4, Table 1–3). Diprenorphine binding affinity was increased twofold at the mu receptor only. Addition of Na^+ was found to increase the affinity of ICI174864 (5.4-fold) at the delta opioid receptor (Table 3).

4PP antagonists displaced radioligand binding in the absence of Na^+ with highest affinity at the human mu opioid receptor followed by the kappa and delta opioid receptors (Tables 1–3). Addition of Na^+ increased the binding affinity of LY255582 by 6-fold at the mu opioid receptor to 0.051 ± 0.006 nM (Fig. 2 and Table 1), 12-fold at the kappa opioid receptor to 0.82 ± 0.14 nM (Fig. 3 and Table 2), and 34-fold at the delta opioid receptor to 4.44 ± 0.52 nM (Fig. 4 and Table 3). Comparison of the rank order of opioid receptor antagonist affinity under low Na^+ conditions established that LY255582 was equipotent with naltrexone at the mu opioid receptor and 10-fold less potent at the kappa and delta opioid receptors. In the presence of Na^+, LY255582 was ~10-fold more potent than naltrexone at the mu opioid receptor and ~2-fold more potent at the kappa and delta opioid receptors. Na^+ addition also enhanced the affinity of (3*R*,4*R*)LY243348, and LY264840, while the binding affinity of the 4PP antagonists LY99335, LY103703, and LY110289 were unaffected or reduced by Na^+ addition (Figs. 1–4 and Tables 1–3). Comparison of the effect of Na^+ on single 4PP isomers was done to further examine the influence of stereochemistry on these effects. In

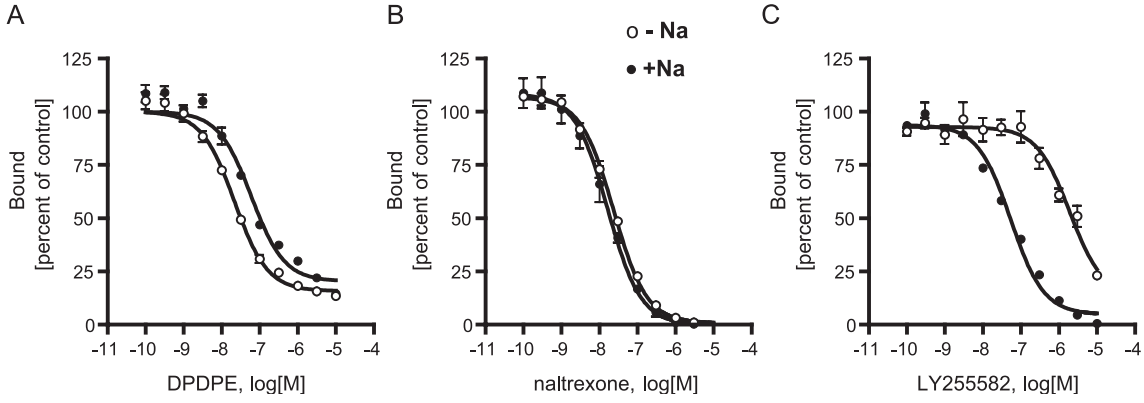

Fig. 4. Sodium effect on DPDPE (A), naltrexone (B), and LY255582 (C) displacement of [^{3}H] bremazocine from CHO-DOR cell membranes. Data are represented as the percent inhibition of specific binding (0.5 nM) [^{3}H] bremazocine in the absence (open symbols) and presence (closed symbols) of 100 mM NaCl. Shown are the results of a representative experiment.

Table 1
Affinity of opioids for the mu opioid receptor in the absence and presence of sodium

Compound	K_i (nM) (+NaCl); mean (S.E.M.)	*n*	K_i (nM) (−NaCl); mean (S.E.M.)	*n*	Na ratio (−Na/+Na)	ED_{20} (mg/kg)[a]
99335	36.16 (8.2)	2	44.2 (5.9)	2	1.22	
103703	25.76 (5.3)	2	35.5 (4.5)	2	1.38	
110289	15.05 (2.2)	2	5.88 (0.86)	2	0.39	
243348	0.077 (0.032)	2	0.62 (0.17)	2	8.08	0.11
243578	0.49 (0.16)	2	1.76 (0.3)	2	3.58	0.4
255265	0.69 (0.08)	2	1.83 (0.17)	2	2.64	>1.25
255609	0.17 (0.06)	2	1.01 (0.44)	2	5.82	0.11
255610	0.35 (0.07)	2	2.48 (0.13)	2	7.07	0.16
255582	0.051 (0.006)	3	0.32 (0.03)	3	6.27	0.04
264840	0.35 (0.08)	2	1.90 (0.34)	2	5.44	0.19
Naltrexone	0.38 (0.03)	21	0.27 (0.04)	13	0.7	
Nalmefene	0.33 (0.05)	2	0.3 (0.1)	2	0.92	
ICI174864	nd		nd			
Naltriben	12.1 (2.9)	4	18.8 (2.4)	4	1.55	
Diprenorphine	0.23 (0.06)	6	0.48 (0.16)	6	2.08	
DPDPE	>1000	2	>1000	2		
DAMGO	15.1 (2.7)	6	1.39 (0.45)	6	0.09	
U69593	>1000	3	304 (37)	3		

K_i values were determined by displacement of [^{3}H]diprenorphine binding in buffer containing Tris–HCl, 5 mM $MgCl_2$, 1 mM EDTA (pH 7.4) in the absence and presence of 100 mM NaCl.
nd: not done.
[a] ED_{20} is the dose of compound producing 20% inhibition of food intake in obese Zucker rats (Mitch et al., 1993).

contrast to (α*S*,3*R*,4*R*)LY255582, the kappa binding affinity of (α*R*,3*S*,4*S*)LY255265 was unaffected by the addition of Na^+ (0.9-fold) and only weakly increased at the mu (2.6-fold) and delta (2.7-fold) receptors. (α*S*,3*S*,4*S*)LY255609 and (α*R*,3*R*,4*R*)LY255610 displayed intermediate increases with Na^+ addition at all three receptors (Tables 1–3). Similarly, (3*S*,4*S*)LY243578 was less sensitive to Na^+ than (3*R*,4*R*)LY243348.

In addition to the enhancement of ICI174864 binding affinity in the presence of Na^+, this peptide has been shown to inhibit the constitutive activity of the delta opioid receptor. The effect of 4PP antagonists on basal GTPγ[^{35}S] binding was measured in delta opioid receptor cell membranes and compared to ICI174864 and other opioids. Consistent with previous reports (Costa and Herz, 1989; Neilan et al., 1999), basal GTPγS binding was sensitive to

Table 2
Affinity of opioids for the kappa opioid receptor in the absence and presence of sodium

Compound	K_i (nM) (+NaCl); mean (S.E.M.)	*n*	K_i (nM) (−NaCl); mean (S.E.M.)	*n*	Na ratio (−Na/+Na)	ED_{20} (mg/kg)[a]
99335	471 (57)	3	417 (25)	3	0.89	
103703	1208 (320)	4	756 (93)	4	0.63	
110289	817 (116)	2	272 (31)	2	0.33	
243348	1.65 (0.38)	4	5.87 (0.77)	4	3.56	0.11
243578	4.29 (0.76)	4	5.23 (0.92)	4	1.22	0.4
255265	7.12 (1.70)	4	6.36 (1.04)	4	0.89	>1.25
255609	2.51 (0.72)	4	6.29 (1.34)	4	2.5	0.11
255610	4.26 (2.17)	3	13.49 (3.47)	3	3.16	0.16
255582	0.82 (0.14)	5	10.3 (2.9)	5	12.56	0.04
264840	3.6 (0.28)	4	12.85 (2.02)	4	3.57	0.19
Naltrexone	2.01 (0.16)	26	1.17 (0.11)	21	0.58	
Nalmefene	0.69 (0.10)	2	0.60 (0.05)	2	0.87	
ICI174864	nd		nd			
Naltriben	24.0 (3.9)	4	17.2 (2.4)	4	0.72	
Diprenorphine	0.23 (0.06)	5	0.25 (0.06)	6	1.09	
DPDPE	>1000	2	>1000	2		
DAMGO	>1000	2	>1000	2		
U69593	4.5 (2.6)	4	3.58 (0.73)	4	0.79	

K_i values were determined by displacement of [^{3}H]diprenorphine binding in buffer containing Tris–HCl, 5 mM $MgCl_2$, 1 mM EDTA (pH 7.4) in the absence and presence of 100 mM NaCl.
nd: not done.
[a] ED_{20} is the dose of compound producing 20% inhibition of food intake in obese Zucker rats (Mitch et al., 1993).

Table 3
Affinity of opioids for the delta opioid receptor in the absence and presence of sodium

Compound	K_i (nM) (+NaCl); mean (S.E.M.)	*n*	K_i (nM) (−NaCl); mean (S.E.M.)	*n*	Na ratio (−Na/+Na)	ED_{20}[a] (mg/kg)
99335	2045 (383)	3	2670 (271)	3	1.31	
103703	1379 (328)	3	2099 (231)	3	1.52	
110289	1533 (79)	2	3326 (810)	2	2.17	
243348	5.46 (0.67)	3	52 (11)	3	9.44	0.11
243578	31.6 (6.4)	3	130 (39)	3	4.1	0.4
255265	169 (27)	4	460 (65)	4	2.73	>1.25
255609	25.6 (7.0)	3	319 (77)	3	12.46	0.11
255610	50 (7)	3	984.1 (42)	3	19.63	0.16
255582	4.44 (0.52)	4	151 (47)	4	34.1	0.04
264840	24.2 (9.4)	3	128 (43)	3	5.29	0.19
Naltrexone	9.98 (0.6)	18	13.36 (0.81)	13	1.34	
Nalmefene	5.6 (1.2)	2	4.8 (0.8)	2	0.85	
ICI174864	38.6 (2.0)	3	209 (33)	3	5.42	
Naltriben	0.35 (0.08)	5	0.40 (0.11)	5	1.14	
Diprenorphine	0.77 (0.22)	6	0.98 (0.28)	6	1.27	
DPDPE	9.9 (3.6)	6	3.21 (0.96)	6	0.32	
DAMGO	692 (307)	2	334 (104)	2	0.48	
U69593	>1000	2	>1000	2		

K_i values were determined by displacement of [^{3}H]bremazocine binding in buffer containing Tris–HCl, 5 mM $MgCl_2$, 1 mM EDTA (pH 7.4) in the absence and presence of 100 mM NaCl.
nd: not done.
[a] ED_{20} is the dose of compound producing 20% inhibition of food intake in obese Zucker rats (Mitch et al., 1993).

Na^+, increasing about twofold when Na^+ was replaced with K^+(data not shown). In the presence of K^+, the delta opioid receptor agonist DPDPE increased GTPγS binding by 116% with an EC_{50} of 12.6 nM, whereas the delta opioid receptor inverse agonist ICI174864 reduced basal GTPγS binding by 62% with an EC_{50} of 294 nM (Fig. 5 and Table 4). Naltrexone and naltriben weakly increased GTPγS binding relative to the stimulation observed with DPDPE (Fig. 5 and Table 4). Examination of the activity of 4PP antagonists revealed that LY255582 inhibited basal GTPγS binding by 57.5% with an EC_{50} of 18.3 nM. Further comparison of the activity of the diastereomers of LY255582 indicated a similar maximum inhibition of basal GTPγS binding and rank order of potency consistent with their observed binding affinities (Fig. 5 and Table 4). The rank order of potency for the opioid receptor antagonists was LY255582>LY255609 = LY255610>ICI174864>LY255265 (Table 4).

The binding of 4PP antagonists was further compared to their previously determined anorectic potency in the obese Zucker rat. The dose of 4PP antagonist required to inhibit food intake by 20% (Mitch et al., 1993) was compared to its binding affinity in the absence and presence of Na^+ (Fig. 6). To eliminate stereochemical effects, compounds used for this comparison were resolved to a single isomer. In the

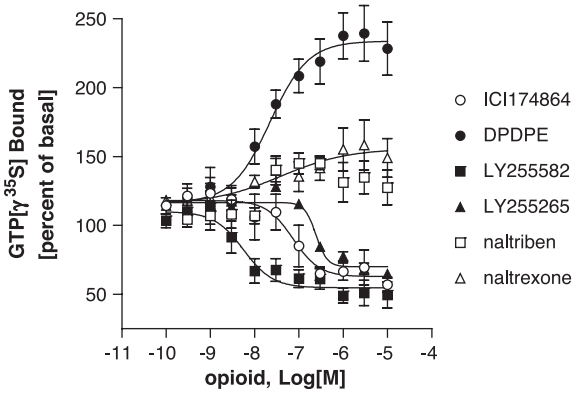

Fig. 5. Opioid effects on basal GTPγ[^{35}S] binding activity in hDOR cell membranes. Values represent the change in specific GTPγ[^{35}S] binding activity in the presence of DPDPE, naltriben, naltrexone, ICI 174864, LY255265, or LY255582. The data shown are from a representative experiment. Data are the mean and S.E.M. from duplicate concentration–response curves.

Table 4
Effect of opioids on basal GTPγS binding in delta opioid receptor membranes

Compound	EC_{50} (nM); mean (S.E.M.)	Percent of basal; mean (S.E.M.)	*n*	ED_{20} (mg/kg)[a]
DPDPE	12.6 (3.9)	+116 (22)	4	
Naltriben	no curve	+22 (7)[b]	6	
Naltrexone	11.3 (9.3)	+46 (6)	4	
ICI-174,864	294 (79)	−62.0 (5.3)	5	
LY255582	18.3 (3.1)	−57.5 (5.3)	6	0.04
LY255609	117 (23)	−58.4 (6.8)	4	0.11
LY255610	119 (14)	−52.5 (6.9)	4	0.16
LY255265	401 (79)	−48.4 (3.9)	5	>1.25

[^{35}S]GTPγS binding was determined as described in Materials and methods in the presence of 100 mM KCl. Results were obtained by sigmoidal curve fit of specific [^{35}S]GTPγS binding as illustrated in Fig. 5.
[a] ED_{20} is the dose of compound producing 20% inhibition of food intake in obese Zucker rats (Mitch et al., 1993).
[b] Data is expressed as the percent of control binding at 10 μM naltriben.

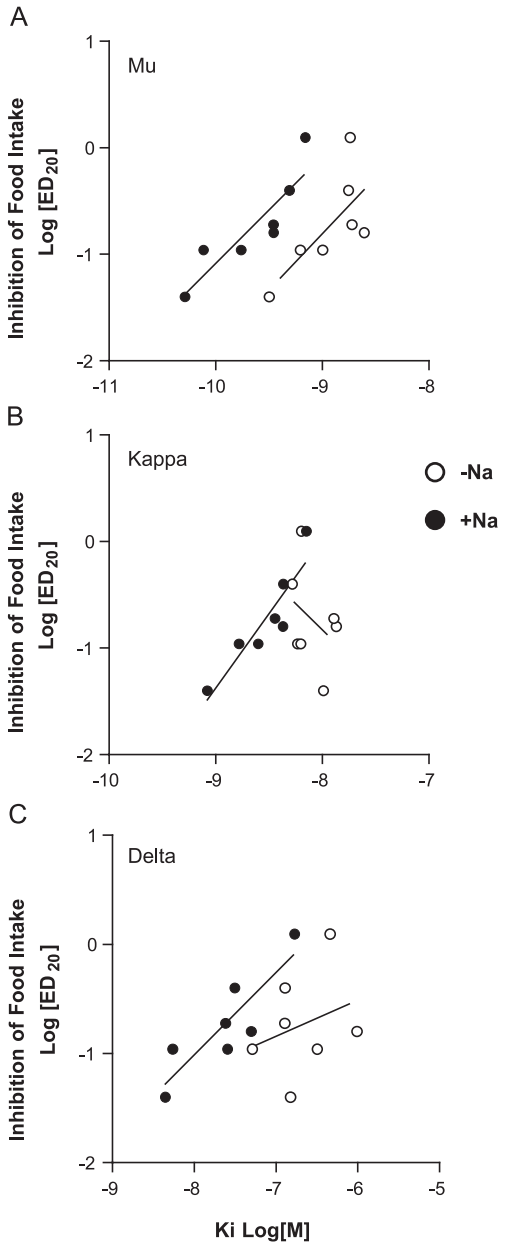

Fig. 6. Correlation between 4PP binding affinity and inhibition of food intake. Receptor binding affinity in the absence (open circles) and presence of 100 mM sodium (closed circles) at the mu (A), kappa (B), and delta (C) receptor are plotted vs. the dose required to reduce food intake by 20% (ED_{20}, mg/kg, s.c.) in obese Zucker rats (Mitch et al., 1993). The compounds used in this figure were LY243348, LY243578, LY255265, LY255582, LY255609, LY255610, and LY264840. The correlation coefficients (r^2) for the lines shown are (A) −Na 0.52, +Na 0.79; (B) −Na 0.13, +Na 0.83; (C) −Na 0.09, +Na 0.76.

absence of Na^+, mu opioid receptor-binding affinity was weakly correlated with anorectic potency ($r^2 = 0.52$), whereas no correlation was observed between kappa ($r^2 = 0.13$) or delta ($r^2 = 0.09$) binding affinity and anorectic potency. However, in the presence of Na^+, mu ($r^2 = 0.79$), kappa ($r^2 = 0.83$), and delta ($r^2 = 0.76$) receptor-binding affinities were found to correlate with anorectic potency.

4. Discussion

4-Phenylpiperidines have been described as potent opioid receptor antagonists with highest affinity at the mu opioid receptor followed by the kappa and delta opioid receptors (Zimmerman et al., 1978; Mitch et al., 1993). Compounds within this series of antagonists have been described as potent anorectics when compared to other morphinan opioid receptor antagonists (Mitch et al., 1993). Furthermore, these antagonists have been shown to exhibit efficacy that is longer lasting than naltrexone (Shaw, 1993). The purpose of the present study was to further characterize the pharmacology of these antagonists in order to identify differences between these classes of opioid receptor antagonists. Whereas morphinan antagonist binding was insensitive to Na^+ and the compounds were neutral antagonists, 4PP binding was enhanced by Na^+ and the compounds displayed inverse agonist activity at the delta opioid receptor.

The influence of cations on opioid receptor binding was characterized soon after the first demonstration of specific opioid binding (Pert and Snyder, 1974). Further characterization of Na^+ effects on opioid receptor antagonists suggested that differences relate to the intrinsic efficacy of these compounds. Binding of the neutral antagonist naltrexone has been shown to be insensitive to Na^+ addition, while the binding affinity of the delta opioid inverse agonists ICI174864 and TIPP(psi) are enhanced by Na^+ (Appelmans et al., 1986, Emmerson et al., 1994, Neilan et al., 1999, Martin et al., 2002). Similar to these findings, we observed that the binding affinities of the morphinan antagonists naltrexone, nalmefene, and naltriben were unaffected by Na^+ addition, whereas the binding affinity of the delta opioid inverse agonist ICI174864 was increased fivefold. Diprenorphine binding to the mu opioid receptor increased twofold in the presence of sodium similar to the effect observed with guanine nucleotide addition (Brown and Pasternak, 1998). The affinity of the mu opioid agonist DAMGO was decreased by Na^+ addition to a greater degree than that of the delta opioid agonist DPDPE, while the kappa opioid agonist U69593 and the nonselective agonist [^{3}H]bremazocine were unaffected by Na^+ addition consistent with previous observations (Clark et al., 1989; Emmerson et al., 1994, Shahrestanifar et al., 1996). Although not studied here, combined addition of Na^+ and GDP has previously been shown to further decrease the binding affinities of U69593 (Remmers et al., 1999), DAMGO (Emmerson et al., 1994), and DPDPE (Clark et al., 1997). The increased affinity of 4PP antagonists in the presence of Na^+ distinguished these antagonists from the morphinan series of opioid receptor antagonists. The effect of Na^+ on the binding of 4PP opioid receptor antagonists was similar to that observed for ICI174864, although it varied greatly between 4PP antagonists and opioid receptors. The most pronounced effects were observed at the delta opioid receptor followed by the mu and kappa opioid receptors. The

larger Na^+ effects observed at the delta opioid receptor may relate to the higher degree of Na^+-sensitive constitutive activity that has been observed relative to the mu opioid receptor (Neilan et al., 1999). LY255582 exhibited the greatest Na^+-induced increases in binding affinity of 34-, 12- and 6-fold at the delta, kappa, and mu opioid receptors. Other antagonists within this series, LY99335, LY103703, and LY110289, were insensitive to Na^+ at all the opioid receptors, suggesting that Na^+ modulation may be dependent on the presence of both the hydroxy-substituted phenyl group and other structural features of the nitrogen substitution. Additionally, the effect of Na^+ on the binding of 4PP antagonists was found to be stereoselective. Na^+ addition had a strong influence on (α*S*,3*R*,4*R*)LY255582 binding but little or no influence on (α*R*,3*S*,4*S*)LY255265 binding and an intermediate influence on (α*S*,3*S*,4*S*)LY255609 and (α*R*,3*R*,4*R*)LY255610 binding. The Na^+ effect was also greater for (3*R*,4*R*)LY243348 than for (3*S*,4*S*)LY243578. The stereoselectivity of these responses suggest that Na^+-induced conformational changes in the receptor (Simon and Groth, 1975) alter the accessibility of ligand–receptor contacts.

Based on the similarity of Na^+ effects on 4PP antagonist and ICI174864 binding affinity, we further examined whether this would be a predictor of inverse agonist activity. The functional activity of opioid receptor antagonists was examined using a GTPγS binding assay. Consistent with previous studies, substitution of Na^+ with K^+ increased the constitutive activity of the delta opioid receptor as indicated by an approximately twofold increase in basal [^{35}S]GTPγS binding (Costa and Herz, 1989; Neilan et al., 1999). In the presence of K^+, ICI174864 and 4PP antagonists were found to reduce basal GTPγS binding by ~ 50%, whereas the morphinan antagonists naltrexone and naltriben increased GTPγS binding weakly compared to the full agonist DPDPE. This effect is similar to that found previously with naloxone at the mu opioid receptor in the absence of Na^+ (Liu and Prather, 2001). Moreover, we found that in the presence of Na^+, naltrexone was without effect, whereas ICI174864 and LY255582 exhibited smaller (~ 25%) but significant decreases in basal GTPγS binding (McKinzie et al., 2001). Although inverse agonist potency was determined in the absence of Na^+ to increase basal GTPγS binding, the rank order of potency was similar to that for delta opioid receptor-binding affinity in the presence of Na^+ (LY255582>LY255609 = LY255610> ICI174864>LY255265). GDP has been shown to have effects similar to Na^+ on delta opioid receptor-G protein coupling (Ott and Costa, 1989). Therefore, the inclusion of GDP in the GTPγS assay buffer would be expected to mimic the effect of Na^+ on 4PP antagonist binding leading to the observed rank order of potency in the functional assay. The class of 4PP inverse agonists was best exemplified by LY255582 where Na^+ increased the delta opioid receptor-binding affinity by 34-fold, and the potency of this compound as an inverse agonist was 16-fold greater than ICI174864. This is in contrast to naltrexone and naltriben which exhibited neither Na^+-potentiated binding nor inverse agonism. Interestingly, regardless of the magnitude of the effect of Na^+ on binding affinity, all the 4PP antagonists tested showed a similar inverse agonist activity. Although LY255265 exhibited only a 2.7-fold increase in binding, this 4PP antagonist decreased basal GTP binding to a similar extent as LY255582. The lack of relation between Na^+ effect and inverse agonist efficacy contrasts with that found for opioid partial agonists where the magnitude of this effect correlates with relative efficacy (Emmerson et al., 1996). Additional compounds in the 4PP antagonist series, RTI-5989-1, -23, and -25, have recently been shown to exhibit inverse agonist activity at the cloned murine delta opioid receptor (Zaki et al., 2001); however, neither the influence of Na^+ on the binding of these antagonists nor their anorectic potency have been reported. Thus, 4PP antagonists differ from classical morphinan antagonists in their response to Na^+ and inverse agonist activity at the delta opioid receptor.

Opioid receptor antagonists have been reported to inhibit food intake in a variety of models (Glass et al., 1999). In rats, naltrexone and naloxone have been shown to inhibit food intake stimulated by the orexigens neuropeptide Y (Kotz et al., 1995), galanin (Dube et al., 1994), and Agouti related peptide (Hagan et al., 2001). In comparison to other opioid receptor antagonists, 4PP antagonists such as LY255582 exhibited greater potency and efficacy in rat models of food intake (Shaw et al., 1990; Levine et al., 1991; Mitch et al., 1993, Shaw, 1993, Statnick et al., 2003). LY255582 produced a statistically significant reduction in food intake in obese Zucker rats over 14 days of treatment, whereas naltrexone exhibited small nonsignificant reductions in food intake over the same period (Shaw, 1993). Naltrexone administration decreased body weight transiently over the initial 5 days of treatment followed by a resumption of weight gain similar to control. In contrast, LY255582 administration decreased body weight throughout 28 (Shaw, 1993) or 68 (Shaw et al., 1991) days of treatment, illustrating that tolerance did not develop to the effects of LY255582. Administration of nalmefene over a 21-day period was found to decrease daily food intake, but body weight was decreased for only the first 7 days (McLaughlin and Baile, 1983). These differences are not likely to be based solely on the relative affinity of 4PP versus morphinan antagonists. LY255582 exhibits higher affinity at only the mu opioid receptor, while LY255609 and LY255610 exhibit binding affinities that are similar to or less than naltrexone and nalmefene (Tables 1–3), yet the 4PP antagonists inhibit food intake with 10-fold greater potency than the morphinan antagonists (Mitch et al., 1993). Additionally, the inhibition of food intake following 4PP administration was not found to clearly correlate with either the mu or the kappa binding affinity measured in rat brain membranes which resulted in uncertainty as to which of the opioid receptor(s) were involved (Levine et al., 1991). To

reexamine this issue, we have compared the binding affinity of a set of 4PP antagonists in the absence and presence of Na^+ to the ED_{20} for inhibition of food intake in the obese Zucker rat model (Mitch et al., 1993). To avoid discrepancies due to differences in the effect of Na^+ on 4PP stereoisomers, only single enantiomers were compared (Mitch et al., 1993). A correlation was observed between binding affinity at all three opioid receptor subtypes determined in the presence of Na^+ and their potency to inhibit food intake ($r^2 \geq 0.76$). In the absence of Na^+, no correlation was observed at the delta or kappa opioid receptors ($r^2 < 0.15$), and a weak correlation was observed at the mu opioid receptor ($r^2 = 0.52$). Although evidence exists for the involvement of all three opioid receptors individually in the control of food intake (Glass et al., 1999), the data presented in this study illustrate a relationship between 4PP antagonist binding affinity in the presence of Na^+ at all three receptors and inhibition of food intake. Because the rank order of 4PP binding affinity is similar at all three opioid receptors, it is difficult to attribute the anorectic effects to activity at any single receptor. However, as evidence exists in the literature for cooperative interactions of opioid receptors in ingestive behaviours (Brugman et al., 2002), the combined occupancy of all three opioid receptor subtypes may explain the greater efficacy of these compounds.

In summary, the 4-phenylpiperidine opioid receptor antagonists exhibit stereospecific enhancement of binding affinity following Na^+ addition at all three opioid receptors and inverse agonist activity at the delta opioid receptor. The inverse agonist activity of 4-phenylpiperidines identifies a specific pharmacological difference between these antagonists and the neutral morphinan antagonists that may underlie the observed differences in anorectic efficacy of these compounds. Furthermore, the observed correlation at all three opioid receptors between inhibition of food intake and 4PP antagonist affinity in the presence of Na^+ suggests that combined opioid receptor occupancy may be required for the potent anorectic effects of these compounds.

References

Appelmans, N., Carroll, J.A., Rance, M.J., Simon, E.J., Traynor, J.R., 1986. Sodium ions increase the binding of the antagonist peptide ICI 174864 to the delta-opiate receptor. Neuropeptides 7, 139–143.

Brown, G.P., Pasternak, G.W., 1998. 3H-Naloxone benzoylhydrazone binding in MOR-1-transfected chinese hamster ovary cells: Evidence for G-protein-dependent antagonist binding. J. Pharmacol. Exp. Ther. 286, 376–381.

Brugman, S., Clegg, D.J., Woods, S.C., Seeley, R.J., 2002. Combined blockade of both mu- and kappa-opioid receptors prevents the acute orexigenic action of Agouti-related protein. Endocrinology 143, 4265–4270.

Cheng, Y.-C., Prusoff, W.H., 1973. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I50) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099–3108.

Clark, M.J., Carter, B.D., Medzihradsky, F., 1989. Selectivity of ligand binding to opioid receptors in brain membranes from the rat, monkey, and guinea pig. Eur. J. Pharmacol. 148, 343–351.

Clark, M.J., Emmerson, P.J., Mansour, A., Akil, H., Woods, J.H., Portoghese, P.S., Remmers, A.E., Medzihradsky, F., 1997. Opioid efficacy in a C6 glioma cell line stably expressing the delta opioid receptor. J. Pharmacol. Exp. Ther. 283, 501–510.

Costa, T., Herz, A., 1989. Antagonists with negative intrinsic activity at delta opioid receptors coupled to GTP-binding proteins. Proc. Natl. Acad. Sci. 86, 7321–7325.

DeLapp, N.W., McKinzie, J.H., Sawyer, B.D., Vandergriff, A., Falcone, J., McClure, D., Felder, C.C., 1999. Determination of [^{35}S]guanosine-5′-*O*-(3-thio)triphosphate binding mediated by cholinergic muscarinic receptors in membranes from Chinese hamster ovary cells and rat striatum using an anti-G protein scintillation proximity assay. J. Pharmacol Exp. Ther. 289, 946–955.

De Ligt, R.A.F., Kourounakis, A.P., Ijzerman, A.P., 2000. Inverse agonism at G protein-coupled receptors: (patho)physiological relevance and implications for drug discovery. Br. J. Pharmacol. 130, 1–12.

Dhawan, B.N., Cesselin, F., Raghubir, R., Reisine, T., Bradley, P.B., Portoghese, P.S., Hamon, M., 1996. International union of pharmacology: XII. Classification of opioid receptors. Pharmacol. Rev. 48, 567–592.

Dube, M.G., Horvath, T.L., Leranth, C., Kalra, P.S., Kalra, S.P., 1994. Naloxone reduces the feeding evoked by intracerebroventricular galanin injection. Physiol. Behav. 56, 811–813.

Emmerson, P.J., Liu, M.R., Woods, J.H., Medzihradsky, F., 1994. Binding affinity and selectivity of opioids at mu, delta and kappa receptors in monkey brain membranes. J. Pharmacol. Exp. Ther. 271, 1630–1637.

Emmerson, P.J., Clark, M.J., Mansour, A., Akil, H., Woods, J.H., Medzihradsky, F., 1996. Characterization of opioid agonist efficacy in a C6 glioma cell line expressing the mu opioid receptor. J. Pharmacol. Exp. Ther. 278, 1121–1127.

Glass, M.J., Billington, C.J., Levine, A.S., 1999. Opioids and food intake: distributed functional neural pathways. Neuropeptides 33, 360–368.

Grudt, T.J., Williams, J.T., 1995. Opioid receptors and the regulation of ion conductances. Rev. Neurosci. 6, 279–286.

Hagan, M.M., Rushing, P.A., Benoit, S.C., Woods, S.C., Seeley, R.J., 2001. Opioid receptor involvement in the effect of AgRP-(83–132) on food intake and food selection. Am. J. Phys. 280, R814–R821.

Ishizuka, Y., Oka, T., 1984. Regulation of opioid antagonist and mu, kappa or delta agonist binding by guanine nucleotide and sodium. Jpn. J. Pharmcol. 36, 397–405.

Kenakin, T., 2001. Inverse, protean, and ligand-selective agonism: matters of receptor conformation. FASEB J. 15, 598–611.

Kotz, C.M., Grace, M.K., Briggs, J., Levine, A.S., Billington, C.J., 1995. Effects of opioid antagonists naloxone and naltrexone on neuropeptide Y-induced feeding and brown fat thermogenesis in the rat. J. Clin. Invest. 96, 163–170.

Levine, A.S., Grace, M., Billington, C.J., Zimmerman, D.M., 1991. Central administration of the opioid antagonist, LY255582, decreases short- and long-term food intake in rats. Brain Res. 566, 193–197.

Liu, J.G., Prather, P.L., 2001. Chronic exposure to mu-opioid agonists produces constitutive activation of mu-opioid receptors in direct proportion to the efficacy of the agonist used for pretreatment. Mol. Pharmacol. 60, 53–62.

Martin, N.A., Ruckle, M.B., VanHoof, S.L., Prather, P.L., 2002. Agonist, antagonist, and inverse agonist characteristics of TIPP (*H*-Tyr–Tic–Phe–Phe–OH), a selective delta-opioid receptor ligand. J. Pharmacol. Exp. Ther. 301, 661–671.

McKinzie, J.H., Emmerson, P.J., Suter, T.M., Surface, P.L., Mitch, C.H., Statnick, M.A., 2001. 3,4-dimethyl-4-(3-hydroxyphenyl)piperidines exhibit sodium dependent opioid receptor binding and inverse agonism. Soc. Neurosci. Abstr. 27, 310.12.

McLaughlin, C.L., Baile, C.A., 1983. Nalmefene decreases meal size, food and water intake and weight gain in Zucker rats. Pharmacol. Biochem. Behav. 19, 235–240.

Miller, R.J., 1998. Presynaptic receptors. Annu. Rev. Pharmacol. Toxicol. 38, 201–227.

Mitch, C.H., Leander, J.D., Mendelsohn, L.G., Shaw, W.N., Wong, D.T., Cantrell, B.E., Johnson, B.G., Reel, J.K., Snoddy, J.D., Takemori, A.E., Zimmerman, D.M., 1993. 3,4-Dimethyl-4-(3-hydroxyphenyl)piperidines: opioid antagonists with potent anorectant activity. J. Med. Chem. 36, 2842–2850.

Neilan, C.L., Akil, H., Woods, J.H., Traynor, J.R., 1999. Constitutive activity of the delta-opioid receptor expressed in C6 glioma cells: identification of non-peptide delta-inverse agonists. Br. J. Pharmacol. 128, 556–562.

Ott, S., Costa, T., 1989. Guanine nucleotide-mediated inhibition of opioid agonist binding. Biochem. Pharmacol. 38, 1931–1939.

Pert, C.B., Snyder, S.H., 1974. Opiate receptor binding of agonists and antagonists affected differentially by sodium. Mol. Pharmacol. 10, 868–879.

Remmers, A.E., Clark, M.J., Mansour, A., Akil, H., Woods, J.H, Medzihradsky, F., 1999. Opioid efficacy in a C6 glioma cell line stably expressing the human kappa opioid receptor. J. Pharmacol. Exp. Ther. 288, 827–833.

Shahrestanifar, M., Wang, W.W., Howells, R.D., 1996. Studies on inhibition of mu and delta opioid receptor binding by dithiothreitol and *N*-ethylmaleimide. J Biol. Chem. 271, 5505–5512.

Shaw, W.N., 1993. Long-term treatment of obese Zucker rats with LY255582 and other appetite suppressants. Pharmacol. Biochem. Behav. 46, 653–659.

Shaw, W.N., Mitch, C.H., Leander, J.D., Zimmerman, D.M., 1990. Effect of phenylpiperidine opioid antagonists on food consumption and weight gain of the obese Zucker rat. J. Pharmacol. Exp. Ther. 253, 85–89.

Shaw, W.N., Mitch, C.H., Leander, J.D., Mendelsohn, L.G., Zimmerman, D.M., 1991. The effect of the opioid receptor antagonist LY255582 on body weight of the obese Zucker rat. Int. J. Obes. 15, 387–395.

Simon, E.J., Groth, J., 1975. Kinetics of opiate receptor inactivation by sulfhydryl reagents: evidence for conformational change in presence of sodium ions. Proc. Natl. Acad. Sci. 72, 2404–2407.

Statnick, M.A., Tinsley, F.C., Eastwood, B.J., Suter, T.M., Mitch, C.H., Heiman, M.L., 2003. Antagonism of opioid receptors reduces body fat in obese rats by decreasing food intake and stimulating lipid oxidation. Am. J. Phys. 284, R1399–R1408.

Vaccarino, A.L., Kastin, A.J., 2001. Endogenous opiates: 2000. Peptides 22, 2257–2328.

Zaki, P.A., Keith Jr., D.E., Thomas, J.B., Carroll, F.I., Evans, C.J., 2001. Agonist-, antagonist-, and inverse agonist-regulated trafficking of the delta-opioid receptor correlates with, but does not require, G protein activation. J. Pharmacol. Exp. Ther. 298, 1015–1020.

Zimmerman, D.M., Nickander, R., Horng, J.S., Wong, D.T., 1978. New structural concepts for narcotic antagonists defined in a 4-phenylpiperidine series. Nature 275, 332–334.