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Na^+-dependent high affinity binding of [^{3}H]LY515300, a 3,4-dimethyl-4-(3-hydroxyphenyl)piperidine opioid receptor inverse agonist

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Abstract

Analogues of 3,4-dimethyl-4-(3-hydroxyphenyl)piperidines are high affinity inverse agonists for μ-, δ- and κ-opioid receptors. To characterize inverse agonist binding, we synthesized a high specific activity radioligand from this series, [^{3}H]LY515300 (3-[1-((3-cyclohexyl-[3,4-$^3H_2$])-3(*R*,*S*)-hydroxypropyl)-3(*R*),4(*R*)-dimethylpiperidin-4-yl]phenol). In membranes expressing cloned human opioid receptors, [^{3}H]LY515300 binding was saturable and exhibited low nonspecific binding. [^{3}H]LY515300 bound with high affinity to the μ- ($K_d = 0.07$ nM), δ- ($K_d = 0.92$ nM) and κ-($K_d = 0.45$ nM) opioid receptors. High affinity [^{3}H]LY515300 binding to all opioid receptors was Na^+-dependent, a characteristic of inverse agonists. Displacement by standard opioid compounds yielded K_i values consistent with their known opioid receptor affinities. Autoradiographic localization of specific [^{3}H]LY515300 binding in rat and guinea pig brain was high in areas known to express high levels of opioid (particularly μ-opioid receptor) binding sites including the caudate, nucleus accumbens, and nucleus tractus solitarius. Thus, [^{3}H]LY515300 is the first radiolabeled opioid receptor inverse agonist useful for the study of opioid receptors in cell lines and native tissues.
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Keywords: μ-Opioid; κ-Opioid; δ-Opioid; 3,4-Dimethyl-4-(3-hydroxyphenyl)piperidine; Antagonist; Receptor autoradiography

1. Introduction

Centrally acting opioids are implicated in the control of numerous physiological responses including analgesia, reward and addiction, appetite and gastrointestinal motility. Opioid peptides produce their effects via G protein-coupled receptors of three distinct classes, μ-, δ-, and κ-opioid receptors (Reisine, 1995; Kieffer, 1995). The cDNAs of each of these receptors have been cloned from numerous species. The deduced amino acid sequences of μ-, δ-, and κ-opioid receptors exhibit high homology among the subtypes and are highly conserved throughout the phylogenetic tree. Recently, a highly related receptor that binds the peptide orphanin FQ/nociceptin was identified by reverse pharmacology of an orphan receptor ORL1 (Meunier et al., 1995; Reinscheid et al., 1995). Opioid receptors signal via pertussis toxin sensitive G_i/G_o proteins to inhibit adenylate cyclase activity (Carter and Medzihradsky, 1993), in addition to activating phospholipase C, G protein-coupled inwardly rectifying K^+ channels and mitogen-activated protein kinases while inhibiting voltage-dependent Ca^{+2} channels (Moises et al., 1994; Kieffer, 1995; Fukuda et al., 1996). Opioid receptors are widely expressed in the central nervous system (CNS) and periphery. Within the CNS, high levels of opioid receptors are found in the prefrontal cortex, striatum, nucleus accumbens, amygdala, median eminence, periaqueductal gray area, nucleus of the solitary tract (NTS) and spinal cord (Mansour et al., 1987; Sharif and Hughes, 1989).

We have previously reported the synthesis of a series of 3,4-dimethyl-4-(3-hydroxyphenyl)piperidine high affinity opioid receptor antagonists (Zimmerman et al., 1993; Mitch et al., 1993). Several compounds of this series exhibit sub-nanomolar binding affinity for the μ- and κ-opioid receptor subtypes, but have lower affinity for the δ-opioid receptor (Mitch et al., 1993; Rothman et al., 1993). Recently, we have reported that 3,4-dimethyl-4-(3-hydroxyphenyl)piperidine opioid receptor antagonists exhibit inverse agonist efficacy in cell lines expressing the cloned human δ-opioid receptor (McKinzie et al., 2001). Structure–activity studies of *N*-substituted phenylpiperi-

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dines indicate that this series retains pure μ- and κ-opioid receptor antagonist activity in vivo, being potent inhibitors of morphine- and *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide (U50, 488)-mediated analgesia, and bremazocine-induced diuresis (Zimmerman et al., 1993; Mitch et al., 1993). Increasing polarity of the *N*-substituent of the 3,4-dimethyl-4-(3-hydroxyphenyl)piperidine restricts compound distribution to the periphery, resulting in opioid antagonists useful in the treatment of gastrointestinal motility disorders (Zimmerman et al., 1994; Schmidt, 2001). Thus, compounds from this chemical series have high in vitro and in vivo potency making them useful tools to study opioid receptor-mediated pharmacology.

Numerous studies implicate the endogenous opioid system in the control of orexigenic behavior. In pre-clinical models of obesity, opioid receptor antagonists reduce food intake and body weight (Margules et al., 1978; Marks-Kaufman et al., 1984; Recant et al., 1980; Apfelbaum and Mandenoff, 1981). Consistent with these findings, treatment with the potent 3,4-dimethyl-4-(3-hydroxyphenyl)piperidine opioid receptor antagonist (3*R*,4*R*)-1-((*S*)-3-hydroxy-3-cyclohexylpropyl)-4-(3-hydroxyphenyl)-3,4-dimethyll-1-piperidine (LY255582) inhibited food intake in obese Zucker rats (Shaw, 1993) and normal Sprague-Dawley rats (Levine et al., 1991). Chronic treatment with LY255582 reduced food intake and body weight gain in obese Zucker rats, which was sustained for 70 days (Shaw et al., 1991). Therefore, blockade of opioid receptors by N-substituted phenylpiperidines may be effective in regulating body weight in humans with clinical obesity.

To evaluate the specificity of 3,4-dimethyl-4-(3-hydroxyphenyl)piperidines for opioid binding sites, we synthesized a ^{3}H-labeled antagonist from this series, 3-[1-((3-cyclohexyl-[3,4-$^3H_2$])-3(*R*,*S*)-hydroxypropyl)-3(*R*),4(*R*)-dimethylpiperidin-4-yl]phenol ([^{3}H]LY515300), which is an equal mixture of the diastereomers LY255582 and (3*R*,4*R*)-1-((*R*)-3-hydroxy-3-cyclohexylpropyl)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidine (LY255610). Here we present the initial characterization of [^{3}H]LY515300 binding to cloned human opioid receptors, and demonstrate the binding of this radioligand to opioid receptors in sections of rat and guinea pig brain.

2. Materials and methods

2.1. Opioid peptides and drugs

LY515300, LY255582 and LY255610 were synthesized following the published methods (Mitch et al., 1991). 5′-Guanidinonaltrindole dihydrochloride (GNTI), 2-(3,4-

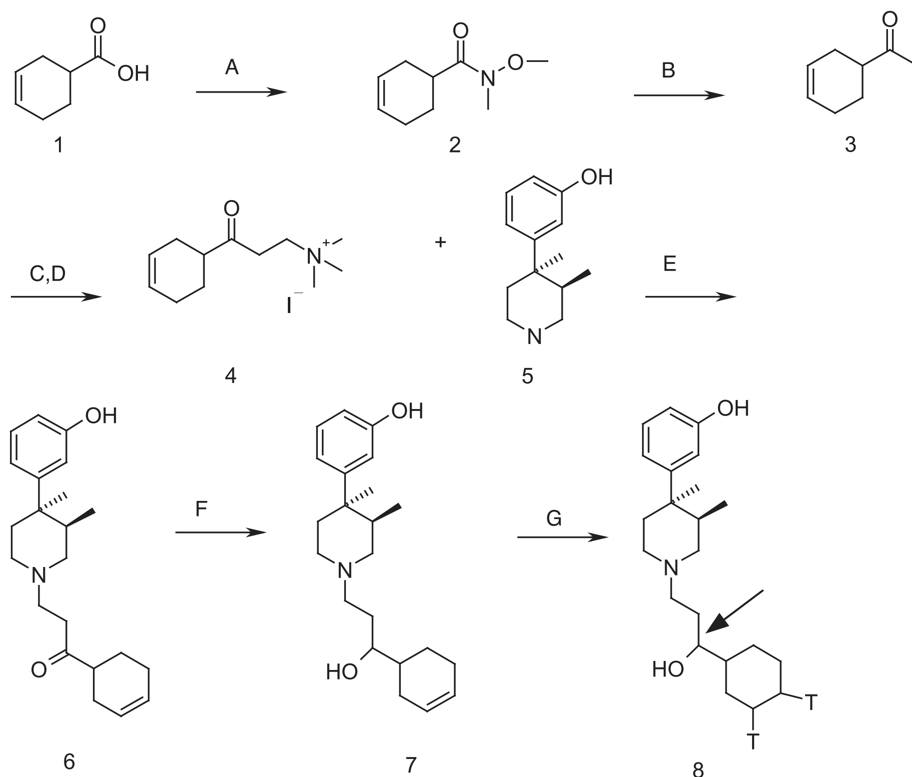

Fig. 1. The chemical structure and synthesis of [^{3}H]LY515300. (A) Isobutyl chloroformate, *N*,*O*-dimethylhydroxylamine; (B) CH_3MgBr; (C) dimethyl amine, $CH_2O_{(x)}$; (D) CH_3I; (E) Na_2CO_3; (F) $NaBH_4$; (G) T_2, 10% palladium on carbon (Pd/C), dimethylformimide. [^{3}H]LY515300 was isolated and used as a mixture of diastereomers at the alcohol stereocenter (indicated by arrow).

dichlorophenyl)-*N*-[(1S)-1-(3-isothiocyanatopheny)-2-(1-pyrrolidinyl)ethyl]acetamide (DIPPA), *N*,*N*-diallyl-Tyr-Aib-Aib-Phe-Leu (ICI 174,864) and nociceptin were purchased from Tocris Cookson (Ballwin, MO). (5α)-17-(Cyclopropylmethyl)-4,5-epoxy-6-methylenemorphinan-3,14-diol (nalmefene), (−)-*N*-(Cyclopropylmehthyl)-4,14-dimethoxymorphinan-6-one hydrobromide (cyprodime), (5α)-17-(Cyclopropylmethyl)-4,5-epoxy-3,14-dihydromorphinan-6-one hydrochloride (naltrexone), *R*(+)-3-(3-hydroxyphenyl)-*N*-propylpiperidine hydrochloride (3-PPP), and (5α)-4,5-epoxy-3,14-dihydro-17-(2-propenyl)morphinan-6-one hydrochloride (naloxone) were purchased from Sigma (St. Louis, MO). (*E*)-4-[[5α,6β)-17-Cyclopropylmethyl)-4,5-epoxy-3,14-dihydroxymorphinan-6-yl]amino]-4-oxo-2-bute-

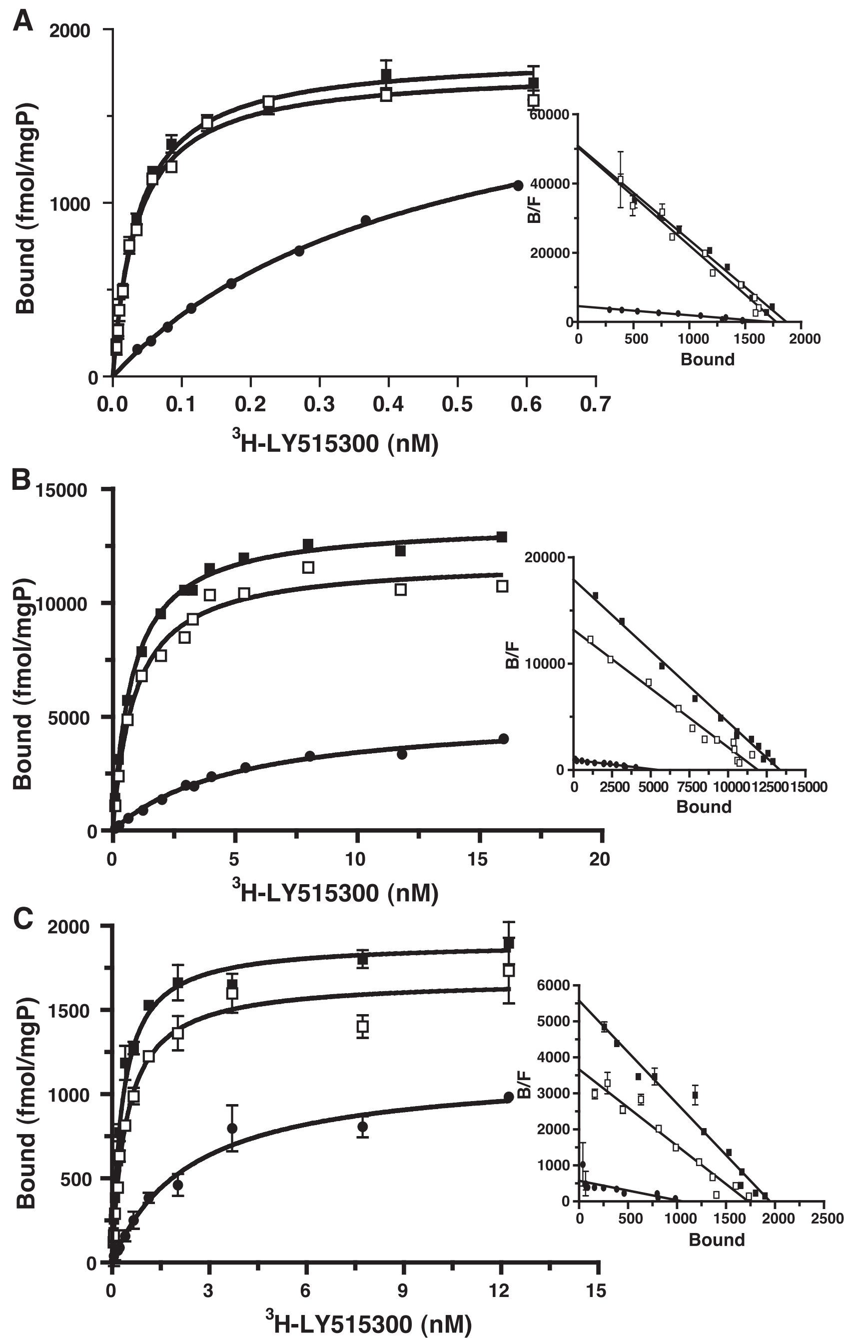

Fig. 2. Saturation binding isotherms and Scatchard plots of [^{3}H]LY515300 binding to CHO membranes expressing cloned human (A) μ-opioid receptor, (B) δ-opioid receptor or (C) κ-opioid receptor. Specific binding was defined as the difference in binding in the absence and presence of 10 μM naloxone. Specific [^{3}H]LY515300 binding was low at all three receptor subtypes in Tris/$MgCl_2$ buffer (filled circle, ●). Binding to all sites was increased by the addition of 100 mM NaCl (open square, □) or 100 mM NaCl and 50 μM GDP (filled square, ■) to the binding buffer. Each data point represents the mean ± S.D. of duplicate determinations determined in duplicate from a single experiment. Binding constants (K_d and B_{max}) were calculated using nonlinear regression analysis from the average of two independent experiments.

Table 1
Effects of sodium and guanine nucleotide on ^{3}H-LY515300 binding to CHO membranes expressing cloned human opioid receptors

Buffer	μ		δ		κ	
	K_d (nM)	B_{max} (fmol/mgP)	K_d (nM)	B_{max} (fmol/mgP)	K_d (nM)	B_{max} (fmol/mgP)
TM	0.32 ± 0.08	1862 ± 748	4.86 ± 0.22	5628 ± 134	1.84 ± 0.28	1115 ± 118
TMN	0.07 ± 0.03[a]	1536 ± 351	0.92 ± 0.02[a]	12,010 ± 130[a]	0.45 ± 0.03[b]	1851 ± 158[b]
TMNG	0.07 ± 0.03[a]	1529 ± 313	0.79 ± 0.04[a]	13,095 ± 330[a]	0.35 ± 0.04[b]	1883 ± 16[b]

Binding constants were calculated using nonlinear regression analysis. Each value represents the mean ± standard deviation of two independent saturation experiments in duplicate. Significantly different from TM buffer.
[a] $P < 0.01$ using two-way ANOVA with Bonferroni post-hoc tests.
[b] $P < 0.05$ using two-way ANOVA with Bonferroni post-hoc tests.

noicacid methyl ester hydrochloride (β-funaltrexamine), 2-(4-ethoxybenzyl)-1-diethylaminoethyl-5-isothiocyanatobenzimidazole hydrochloride (benzimidazole isothiocyanate, BIT), 17-(Cyclopropylmethyl)-6,7-dehydro-4,5α-epoxy-3,14-dihydroxy-6,7-2′, 3′-indolomorphinan hydrochloride (naltrindole), and 17,17′-(Dicyclopropylmethyl)-6,6′,7,7′-6,6′-imino-7,7′-binorphinan-3,4′,14,14′-tetrol dihydrochloride (nor-BNI) were purchased from Research Biochemicals International (Natick, MA). All other reagents were of the highest grade commercially available.

2.2. Synthesis of [^{3}H]LY515300

Fig. 1 outlines the chemical structure and synthesis of [^{3}H]LY515300. The precursor for tritiation, 3-[1-(3-cyclohex-3-enyl-3(*R*,*S*)-hydroxy-propyl)-3(*R*), 4(*R*)-dimethyl-piperidin-4-yl]-phenol, was prepared according to previously reported methods (Mitch et al., 1991). The unsaturated alcohol precursor molecule (5.9 mg) was dissolved in dimethylformamide, treated with 10% palladium on carbon (Pd/C) (6.2 mg), and stirred under an atmosphere of tritium gas (T_2, 10 Ci) for 2 h. The catalyst was removed by filtration and the mixture was evaporated in vacuo. The residue was re-dissolved in ethanol and evaporated; this procedure was repeated several times to remove the labile tritium. The residue was purified by gradient elution high performance liquid chromatography on a Vydac Protein and Peptide C18 column (4.6 × 250 mm) at 1 ml/min (Solvent A, 0.1% aqueous trifluroacetic acid; Solvent B, 0.1% trifluroacetic acid in acetonitrile; 15–45% B over 45 min) with UV detection at 214 nm as well as radiochemical detection, to yield [^{3}H]LY515300 (3-[1-((3-cyclohexyl-[3,4-$^3H_2$])-3(*R*,*S*)-hydroxypropyl)-3(*R*),4(*R*)-dimethylpiperidin-4-yl]phenol), as an equal mixture of the diastereomers LY255582 and LY255610 at the alcohol stereocenter. The individual isomers: LY255582 (3*R*,4*R*)-1-((*S*)-3-hydroxy-3-cyclohexylpropyl)-4-(3-hydroxyphenyl)-3,4-dimethyll-1-piperidine and LY255610 (3*R*,4*R*)-1-((*R*)-3-hydroxy-3-cyclohexylpropyl)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidine, have been previously described (Mitch et al., 1993). The specific activity of [^{3}H]LY515300 (as determined by mass spectrometry) was 64 Ci/mmol.

2.3. Receptor binding of [^{3}H]LY515300 to cloned human opioid receptors

Saturation, kinetic and competition binding experiments were evaluated in fraction P2 membranes isolated from Chinese hamster ovary (CHO) cells expressing cloned human μ-, δ- or κ-opioid receptors (Receptor Biology, Beltsville, MD). Receptor binding assays were carried out using 5–8 μg

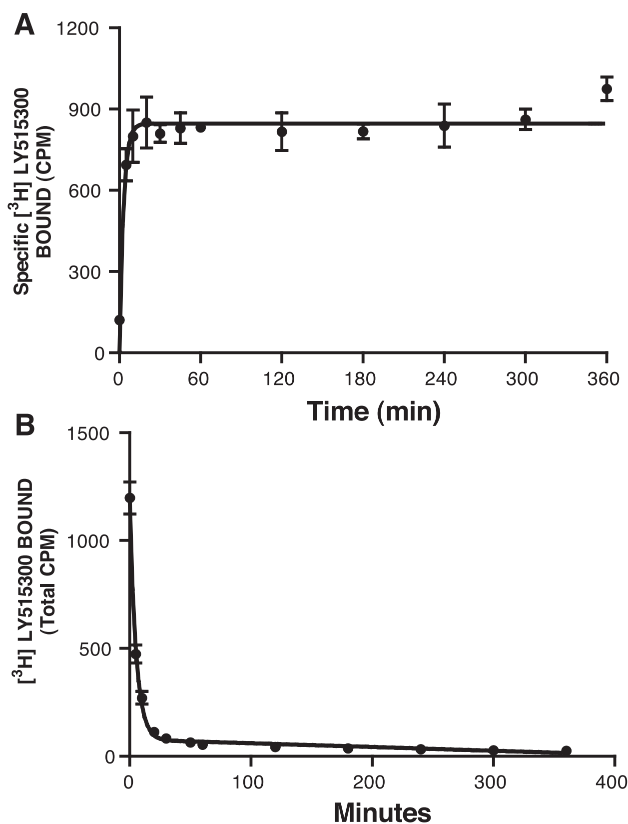

Fig. 3. Time course for the association (A) and the dissociation (B) of [^{3}H]LY515300 (1.1 nM) binding to CHO membranes expressing the cloned human δ-opioid receptor. Association was initiated by the addition of [^{3}H]LY515300 to membranes at different times before filtration. Dissociation studies allowed binding of [^{3}H]LY515300 to reach steady-state for 90 min prior to the addition of 100 nM LY255582 at different times before filtration. Kinetic constants were determined from nonlinear regression analysis using GraphPad Prism. Each data point represents the mean ± S.D. for two independent experiments on triplicate samples.

Table 2
Estimated kinetic constants of ^{3}H-LY515300 binding to CHO membranes expressing cloned human opioid receptors

	μ	δ	κ
K_{ob} (min^{-1})	0.041 ± 0.003	0.4 ± 0.09	0.22 ± 0.01
K_{off} (min^{-1})	0.008 ± 0.0005	0.2 ± 0.01	0.12 ± 0.03
K_{on} (M^{-1}, min^{-1})	0.3e^8	0.19e^8	0.22e^8
K_d (nM)	0.03	1.0	0.54

Kinetic estimates calculated using nonlinear regression analysis. Values represent the mean ± standard deviation of two independent experiments in duplicate.

membranes per well in 50 mM Tris–HCl (pH7.4), 5 mM $MgCl_2$ alone (TM) or containing either 100 mM NaCl (TMN) or 100 mM NaCl, 50 μM GDP (TMNG). Nonspecific binding was determined in the presence of 10 μM naloxone or unlabeled LY255582. Reactions were incubated in duplicate at for 90 min, which permitted the assay to reach equilibrium. Dissociation rate constants (K_{off}) were determined in Tris–HCl/$MgCl_2$/NaCl buffer after addition of 100 nM unlabeled LY255582 at different times before filtration to membranes incubated with [^{3}H]LY515300 (0.11, 1.1 and 0.45 nM at μ-, δ- and κ-opioid receptors, respectively). The time course for estimating association rate constants (K_{on}) was measured in Tris–HCl/$MgCl_2$/NaCl buffer following the addition of [^{3}H]LY515300 (0.11, 1.1 and 0.45 nM at μ-, δ- and κ-opioid receptors, respectively) to membranes at different times before filtration. Bound radioligand was separated from free by rapid filtration through FilterMat type A (Wallac, Turku, Finland) presoaked in 50 mM Tris–HCl pH 7.4 containing 0.05% polyethyleneimine using a 96-well Tomtec cell harvester. The filters were then washed three times with 5 ml Tris–HCl, pH 7.5 at 4 °C. Filters were dried for 90 min at 60 °C, imbedded with MeltiLex A solid scintillant (Wallac) and counted using a Wallac Microbeta. Binding data were analyzed using nonlinear regression analysis to obtain kinetic constants with GraphPad Prism. Competition curves were used to generate an IC_{50} displacing 0.1, 0.4 or 1 nM of [^{3}H]LY515300 in Tris–HCl/$MgCl_2$/NaCl buffer at μ-, δ- and κ-opioid receptor expressing membranes, respectively. The K_i was calculated from IC_{50}s using the equation of Cheng and Prusoff (1973) $K_i = IC_{50}/(1 + R/K_d)$ where R equals the radioligand concentration and K_d equals the binding affinity for the radioligand. All values represent the mean of three independent experiments.

2.4. Autoradiography of [^{3}H]LY515300 binding in rat and guinea pig brain

Male, Sprague-Dawley rats (300–350 g, Harlan, Indianapolis, IN) and male, Hartley guinea pigs (400–450 g, Harlan) were sacrificed by rapid decapitation. Their brains were then removed, chilled in cold phosphate-buffered saline and frozen at −70 °C until used for sectioning. Twelve-micrometer coronal sections of rat and guinea pig brains were thaw mounted onto chromium alum/gelatin coated slides. The

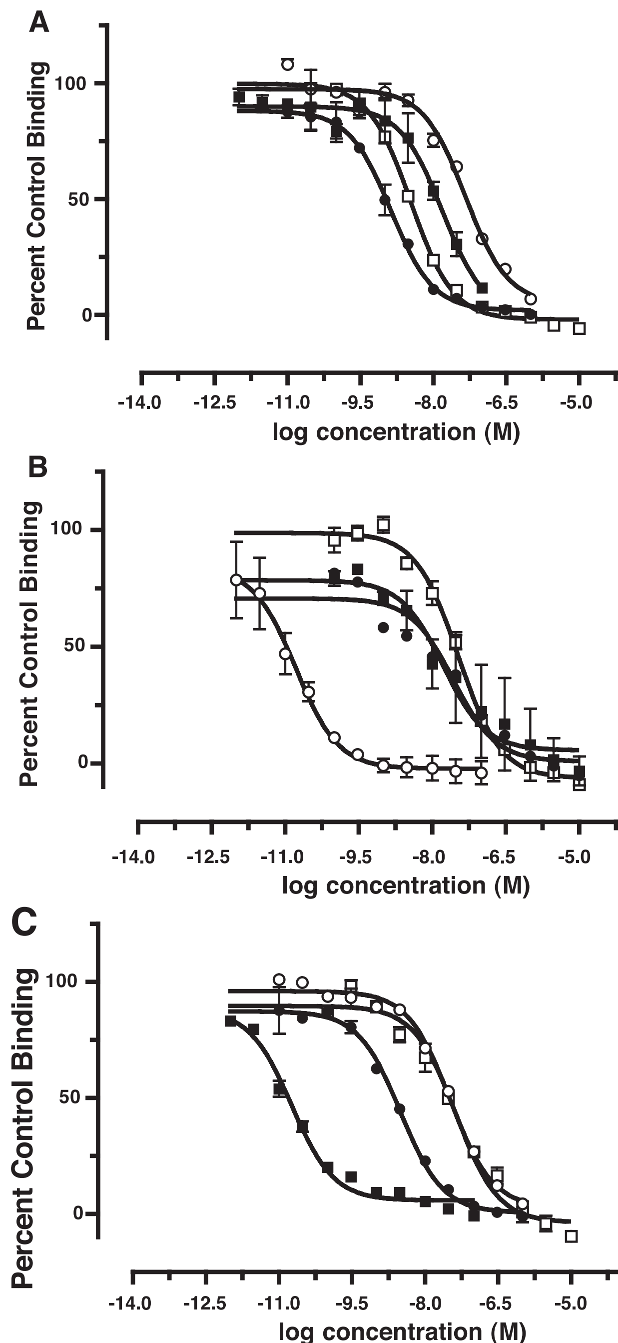

Fig. 4. Pharmacology of [^{3}H]LY515300 binding to CHO membranes expressing cloned human μ-, δ- or κ-opioid receptors. Inhibition curves for naltrexone (●), nor-binaltorphimine (■), naltrindole (○) and β-funaltrexamine (□) were generated against 0.15, 1, or 0.4 nM [^{3}H]LY515300 for μ-opioid receptor (A), δ-opioid receptor (B) or κ-opioid receptor (C), respectively, by varying the concentration of unlabeled test compound. The K_i was calculated from resulting IC_{50}s using the equation of Cheng and Prusoff (1973) $K_i = IC_{50}/(1 + R/K_d)$ where R equals the radioligand concentration and K_d equals the binding affinity for the radioligand. All data points represent the mean of three independent experiments determined in duplicate.

sections were stored at − 20 °C overnight and then moved to − 70 °C until used in the assay. Sections were pre-incubated in a modified Kreb's phosphate buffer containing 0.05% bacitracin and 0.4% bovine serum albumin for 30 min at room temperature. Incubations were conducted in the presence of 1 nM [^{3}H]LY515300 for 1 h at room temperature. Nonspecific binding was determined using a combination of benzimidazole isothiocyanate, naltrindole, DIPPA and (+)*R*-3-PPP at 1 μM each, or 1 μM LY255582 in the incubation buffer containing an adjacent set of sections. Sections were rinsed 2 × 10 min in ice-cold buffer without radioligand, bacitracin or bovine serum albumin, dipped once in cold distilled H_2O and then dried quickly with a cool stream of air. Labeled sections were then placed against a sheet of [^{3}H]-Hyperfilm (Amersham) for 3 weeks. The film was developed using Kodak D-19 developer and fixed using Rapid-Fix (Kodak). The resulting autoradiograms were analyzed using MCID (Imaging Research, St. Catherines, Canada). For displacement curve assays 12 μm coronal sections at the level of the rat caudate (*n* = 3) were incubated with various concentrations of benzimidazole isothiocyanate with the exception that the sections were not dried after the assay. In these experiments labeled sections were wiped from the slide using Whatman 540, 2.1 cm filter circles and placed into scintillation vials. Scintillation cocktail was added to each vial and the radioactivity bound to the tissue assessed using a scintillation counter. Data analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA).

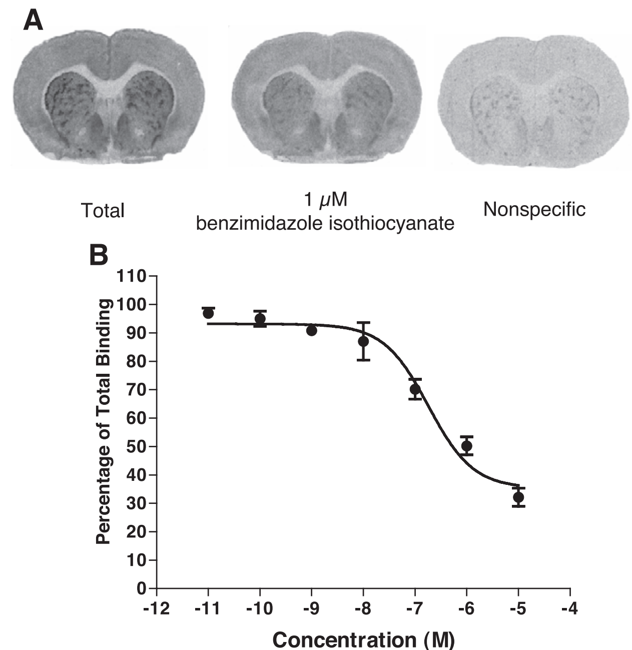

Fig. 5. (A) Distribution of [^{3}H]LY515300 binding in the rat brain. From left to right, total binding, binding in the presence of 1 μM benzimidazole isothiocyanate and binding in the presence of benzimidazole isothiocyanate, naltrindole, DIPPA and (+)R-3-PPP each at 1 μM. (B) Displacement curve of benzimidazole isothiocyanate in 12 μm sections corresponding to the autoradiograms seen in (A). Labeled sections were wiped from the slide using small circular filters, placed in scintillation vials and counted as described in Materials and methods. Nonlinear regression analysis produced a calculated EC_{50} = 181 nM. All data points represent the mean of three independent experiments.

3. Results

3.1. Binding kinetics and pharmacology in membrane homogenates

Specific binding of [^{3}H]LY515300 to cloned opioid receptors was linear with protein and exhibited >90% specific binding. Saturation binding to CHO membranes expressing cloned human μ-, δ- and κ-opioid receptors was saturable (Fig. 2). Nonlinear regression analysis indicated that a single class of binding sites was labeled at each of the receptor subtypes. Interestingly, binding of [^{3}H]LY515300 displayed Na^+ and GDP dependence (Fig. 2). Significant increases in receptor affinity (4.6-, 6.1- and 4.1-fold at μ-, δ- and κ-opioid receptors, respectively) were observed following the addition of 100 mM NaCl to the Tris–HCl/$MgCl_2$ buffer (Table 1). The number of receptors labeled (B_{max}) increased to a lesser degree, but exhibited significant increases in membranes expressing either δ- or κ-opioid receptor subtypes under high Na^+ conditions (2.1- and 0.64-fold, respectively). Compared with ^{3}H-diprenorphine, receptor density using [^{3}H]LY515300 was 1.5 vs. 1.5 pmol/mgP at μ, and 1.8 Vs 2.5 pmol/mgP at κ (data not shown). Similarly, [^{3}H]LY515300 and [^{3}H]bremazocine generated comparable receptor densities of 12 and 10.6 pmol/mgP, respectively, in membranes expressing the δ-opioid receptor (data not shown). Tris–HCl/$MgCl_2$/NaCl/GDP buffer modestly enhanced the estimated binding constants over Tris–HCl/$MgCl_2$/NaCl buffer alone in membranes expressing κ- and δ-opioid receptors, while no difference between these buffers was observed on μ-opioid receptor binding (Table 1).

Table 3
Pharmacology of ^{3}H-LY515300 binding in membranes expressing recombinant human opioid receptor subtypes

Compound	K_i (nM)		
	μ	δ	κ
Naltrexone	0.46 ± 0.06	18.5 ± 3.6	1.7 ± 0.05
Naloxone	6.2 ± 0.6	32.0 ± 3.6	13.3 ± 0.7
Nor-binaltorphimine	5.31 ± 0.13	11.3 ± 2.2	0.01 ± 0.006
Naltrindole	15.8 ± 0.8	0.011 ± 0.001	17.7 ± 0.06
Cyprodime	22.6 ± 6.0	826.1 ± 147.0	188.8 ± 57.0
GNTI	21.8 ± 7.0	20.8 ± 5.0	0.04 ± 0.002
β-Funaltrexamine	1.1 ± 0.29	17.6 ± 4.7	20.6 ± 7.0
ICI-174,864	>1000	21.4 ± 9.8	>1000
Nalmefene	0.6 ± 0.06	9.9 ± 0.5	1.0 ± 0.06
Nociceptin	>1000	>1000	>1000
LY255582	0.08 ± 0.005	2.0 ± 0.09	0.48 ± 0.05
LY255610	0.43 ± 0.05	6.40 ± 0.06	2.16 ± 0.07

K_i values were calculated from IC_{50}s using the equation of Cheng and Prusoff (1973). Each value represents the mean ± S.E.M. of three independent experiments.

Association of specific [^{3}H]LY515300 binding in Tris–HCl/$MgCl_2$/NaCl buffer to human μ-, δ- and κ-opioid receptor expressing membranes was time dependent, and reached steady-state at approximately 90 min. Nonlinear regression analysis of [^{3}H]LY515300 binding best fit a one-phase association model for all opioid

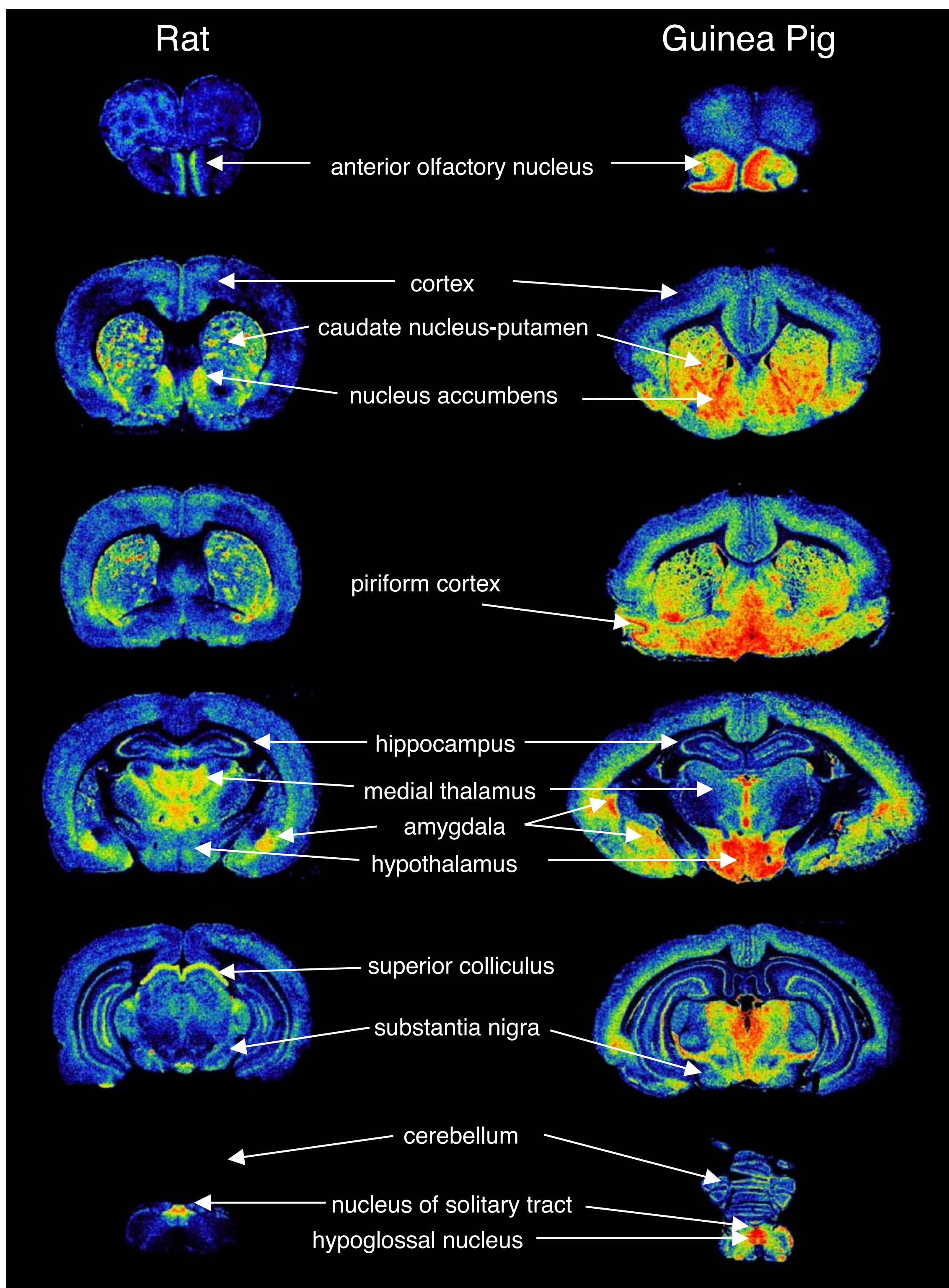

Fig. 6. Distribution of [^{3}H]LY515300 binding in rat and guinea pig brain. Sections of rat and guinea pig brain were incubated with 1 nM [^{3}H]LY515300 alone or in the presence of 1 μM LY255582. Incubation in the presence of 1 μM LY255582 resulted in nonspecific binding that was equal to film background (not shown). Areas of high binding appear in red to yellow pseudocolor.

receptor subtypes (Fig. 3A and Table 2). Specific [^{3}H]LY515300 binding in Tris–HCl/$MgCl_2$/NaCl buffer dissociated in the presence of unlabeled LY255582. The kinetics for dissociation was best fit to a one-phase model (Fig. 3B and Table 2). The dissociation constant (K_d) determined from the ratio of K_{off}/K_{on} was 0.03, 1.0 and 0.54 nM at μ-, δ- and κ-opioid receptors, respectively (Table 2). These values were comparable to the apparent K_d values (0.07, 0.92 and 0.45 nM at μ-, δ- and κ-opioid receptors, respectively) calculated from saturation binding studies conducted in Tris–HCl/$MgCl_2$/NaCl buffer (Table 1).

The pharmacology of [^{3}H]LY515300 binding at the cloned human μ-, δ- and κ-opioid receptors was evaluated by displacement of known opioid receptor ligands. Displacement of [^{3}H]LY515300 binding to μ-, δ- and κ-opioid receptors with antagonists exhibited expected selectivity profiles and produced K_i values and rank orders of potency consistent with literature values (Fig. 4 and Table 3). The structurally related 4-hydroxyphenylpiperidines, LY255582 and LY255610, demonstrated high affinity binding to μ-, δ- and κ-opioid receptors, with K_i values consistent with those produced when the sites were labeled with [^{3}H]diprenorphine (μ- and κ-opioid receptors) or with [^{3}H]bremazocine (δ-opioid receptor) (data not shown). The opioid-related peptide nociceptin was ineffective at displacing [^{3}H]LY515300 binding at the opioid receptors tested, consistent with this peptide having high affinity for the ORL1 receptor and low affinity for classical opioid receptors (Table 3). Naltrindole, ICI-174,864, exhibited approximately 1000-fold selectivity for δ-opioid binding sites, while nor-binaltorphimine and GNTI had similar selectivity for κ-opioid binding sites labeled with [^{3}H]LY515300. No compounds displaced [^{3}H]LY515300 binding with high selectivity at the μ-opioid receptor. The rank order of potency for binding to μ-opioid binding sites was LY255582 > LY255610 ≥ naltrexone = nalmefene > β-funaltrexamine > nor-binaltorphimine = naloxone > naltrindole = cyprodime = GNTI ≫ ICI-174,864 = nociceptin. For all tested compounds, specific [^{3}H]LY515300 binding was inhibited with uniform Hill slopes (0.75–1), describing a single class of binding sites.

3.2. Binding site distribution in rat and guinea pig brain measured by receptor autoradiography

To evaluate the utility of this radioligand to label opioid receptors in native tissues, we examined the distribution of [^{3}H]LY515300 binding sites in rat and guinea pig brain using receptor autoradiography. In both rat and guinea pig brain, nonspecific binding was low to moderate under our conditions. When incubated in the presence of the opioid antagonist benzimidazole isothiocyanate, [^{3}H]LY515300 binding to striatal sections was reduced in a dose-dependent manner (Fig. 5). Approximately 30% residual binding of [^{3}H]LY515300 was not displaced by 10 μM benzimidazole isothiocyanate and was localized in the striatal matrix and nucleus accumbens, with lower amounts found in the frontal cortex (Fig. 5). The distribution of [^{3}H]LY515300 binding in rat and guinea pig brain is presented in Fig. 6. In the rat, the highest density of binding was found in patches of the caudate, nucleus accumbens, amygdaloid nuclei, mediodorsal nucleus of the thalamus, zonal and superficial gray layers of the superior colliculus, area postrema and the nucleus of the solitary tract. Moderate levels were seen in the cortex, matrix of the caudate, several thalamic nuclei, CA3 (oriens layer) and molecular layer of the hippocampus, hypothalamus, central gray and spinal trigeminal tract. Low levels were noted in the dorsal lateral septum, medial geniculate, reticulata of the substantia nigra, hypoglossal nucleus and cerebellum. The guinea pig brain had the highest density of binding in the accessory olfactory nucleus, patches in the caudate, nucleus accumbens, amygdala, piriform cortex, central medial and reuniens thalamic nuclei, several nuclei in the hypothalamus, peripeduncular nucleus, area postrema, nucleus of the solitary tract and hypoglossal nucleus. Moderate levels were seen in the cortex, dorsal lateral septum, CA1, CA2 and CA3 (oriens layer) of the hippocampus, substantia nigra pars reticulata, central gray, spinal trigeminal tract and molecular layer of the cerebellum. The areas containing the lowest density of binding in the guinea pig were the rostral thalamus and medial geniculate. Therefore, in both rat and guinea pig brain [^{3}H]LY515300 bound to sites exhibiting a distribution consistent with known opioid receptors in these species.

4. Discussion

Mitch et al. (1991) have reported that 3,4-dimethyl-4-(3-hydroxyphenyl)piperidine analogs are high affinity opioid receptor antagonists. Recently, we demonstrated that 3,4-dimethyl-4-(3-hydroxyphenyl)piperidine antagonists exhibit inverse agonist efficacy at the cloned human δ-opioid receptor, inhibiting basal GTPγS binding in a naltrexone reversible manner (McKinzie et al., 2001). In competition binding studies using rat or guinea pig brain membranes, LY255582 is reported to have high affinity for μ-opioid binding sites (K_i = 0.41 nM), with lower affinity for κ- and δ-opioid binding sites (K_i values of 2 and 5.2 nM, respectively). Structure–activity studies of *N*-substituted phenylpiperidines indicate that this series retains pure μ- and κ-opioid receptor antagonist activity in vivo with no agonist efficacy (Zimmerman et al., 1993; Mitch et al., 1993). In the present study, we examined the binding properties of [^{3}H]LY515300 in membrane homogenates from cells expressing cloned human opioid receptors and in sections of rat and guinea pig brain. From these data, we have concluded that [^{3}H]LY515300 is a high affinity inverse agonist radioligand for μ-, δ- and κ-opioid receptors with high specific activity and low nonspecific binding.

Binding of [^{3}H]LY515300 to membrane homogenates of cloned μ-, δ- or κ-opioid receptors was saturable and reversible. Nonspecific binding, as defined by 1 μM naltrexone, was < 10% in membrane homogenates. The estimated B_{max} for [^{3}H]LY515300 binding in Tris–HCl/$MgCl_2$/NaCl at each receptor clone was consistent with values obtained when labeling with the opioid antagonists [^{3}H]diprenorphine or [^{3}H]bremazocine. The association kinetics and dissociation kinetics of [^{3}H]LY515300 binding in Tris–HCl/$MgCl_2$/NaCl were best fit to one-phase models in membranes expressing μ-, δ- or κ-opioid receptors. Therefore, a single class of binding site was labeled in each of the membrane preparations with this antagonist. Furthermore, the K_{off}/K_{on} ratio estimated affinities for μ-, δ- and κ-opioid binding sites that were consistent with the estimated K_d values from saturation studies in Tris–HCl/$MgCl_2$/NaCl buffer.

3,4-Dimethyl-4-(3-hydroxyphenyl)piperidine antagonists have high affinity for μ-, δ- and κ-opioid receptors in membrane homogenates from cells expressing cloned human opioid receptors. We found that the affinities of reference opioid receptor ligands were consistent with previously reported values (Mitch et al., 1991; Emmerson et al., 1994; Schmidhammer et al., 1995; Clark et al., 1997; Remmers et al., 1999; Broadbear et al., 2000; Jones and Portoghese, 2000). Naltrindole and ICI-174,864 exhibited approximately 1000-fold selectivity for δ-opioid binding sites, while nor-binaltorphimine and GNTI exhibited similar selectivity for κ-opioid binding sites vs. other opioid sites labeled with [^{3}H]LY515300. No compounds displaced [^{3}H]LY515300 binding with comparable selectivity at μ-opioid binding sites. Under our assay conditions, β-funaltrexamine was approximately 20-fold μ-selective over δ- and κ-opioid binding sites, while cyprodime was only 10-fold μ-selective vs. κ-opioid binding sites and 35-fold μ-selective vs. δ-opioid binding sites. Our findings were consistent with previous reports on the affinity and selectivity of β-funaltrexamine (Corbett et al., 1985; Broadbear et al., 2000) and cyprodime (Schmidhammer et al., 1990, 1995) under low Na^+ agonist binding conditions at opioid binding sites in mouse and guinea pig brain membranes. An important note was that our inhibition curves were generated against 0.1, 1 or 0.4 nM [^{3}H]LY515300 in membranes expressing μ-, δ- or κ-opioid receptors, respectively. Therefore, the possibility exists that selective labeling of opioid receptor subtypes could be obtained with [^{3}H]LY515300 (particularly at the μ-opioid receptor) by utilizing appropriate ligand concentrations and blocking concentrations of unlabeled receptor selective antagonists, but this hypothesis needs further evaluation.

In rat and guinea pig brain sections, the distribution of [^{3}H]LY515300 binding was consistent with findings using the nonselective opioid receptor ligands [^{3}H]diprenorphine or [^{3}H]bremazocine and [^{3}H]ethylketocyclazocine in these species, respectively (Pert and Snyder, 1974; Foote and Maurer, 1982; Quirion et al., 1983). High-density patch-like binding of [^{3}H]LY515300 was observed in the striatum of rat and guinea pig, which was inhibited in the rat in a dose-dependent manner by the μ-opioid selective compound benzimidazole isothiocyanate. Similar striatal patches initially identified using [^{3}H]diprenorphine labeling (Atweh and Kuhar, 1977b) were found to contain μ-opioid receptor binding sites (Mansour et al., 1987; Sharif and Hughes, 1989). Approximately 30% of the [^{3}H]LY515300 binding in striatal patches was not displaced with 10 μM of the μ-opioid receptor antagonist benzimidazole isothiocyanate, suggesting that residual μ-opioid receptor binding or binding to other receptor sites was present under these conditions. Interestingly, binding in non-patch regions of the striatum and nucleus accumbens was reduced to a lesser degree by benzimidazole isothiocyanate. This is consistent with these regions containing significant levels of δ- and κ-opioid binding sites (Mansour et al., 1987; Sharif and Hughes, 1989). Alternatively a portion of this binding may represent residual μ-opioid receptor binding or binding to other non-opioid receptor sites, since this binding was not observed in brain sections incubated with blocking concentrations of LY255582 or a combination of benzimidazole isothiocyanate, naltrindole, DIPPA and (+)-3PPP. Consistent with earlier reports describing opioid binding sites using nonselective opioid receptor antagonists (Wamsley, 1983), high [^{3}H]LY515300 binding was found in the rat and guinea pig nucleus accumbens, basolateral nucleus of the amygdala, interpeduncular nucleus, various thalamic nuclei, area postrema and the nucleus of the solitary tract. Similar to the striatum, these regions were found to exhibit high μ-opioid receptor expression (Mansour et al., 1987; Sharif and Hughes, 1989). Several notable species differences consistent with the known distributions of opioid receptors in the rat and guinea pig brain were identified using [^{3}H]LY515300. High to moderate binding levels of [^{3}H]LY515300 binding were found in the guinea pig anterior olfactory nucleus, piriform cortex, hypothalamus and cerebellum, while these regions displayed significantly lower [^{3}H]LY515300 binding in the rat. Similar species differences were reported using the nonselective opioid receptor radioligands [^{3}H]diprenorphine [^{3}H]bremazocine or [^{3}H]ethylketocyclazocine (Atweh and Kuhar, 1977a,b; Foote and Maurer, 1982; Palacios and Maurer, 1984; Quirion et al., 1983). Preferential binding in the guinea pig brain most likely represents binding to κ-opioid receptors, which are expressed at much higher levels in the guinea pig brain compared to the rat brain, as evidenced by dense localization of κ-opioid receptor mRNA, binding sites and agonist stimulation of GTPγS binding (Robson et al., 1984; Itzhak et al., 1984; Sharif and Hughes, 1989; Xie et al., 1994; Sim and Childers, 1997). Conversely, [^{3}H]LY515300 binding was higher in the rat thalamus and superior colliculus than in the guinea pig, while

moderate to high levels of binding were found in the diagonal band of brocca, cortex, CA1, CA2 and CA3 (oriens layer) of the hippocampus, and nucleus of the solitary tract in both the rat and guinea pig, consistent with the known distribution of opioid binding sites in these species (Atweh and Kuhar, 1977a,b; Foote and Maurer, 1982; Quirion et al., 1983). Further studies using masking agents and differing concentrations of radioligand are presently underway in order to characterize the pharmacology of [^{3}H]LY515300 binding sites. Thus, consistent agreement with other nonselective radioligands in the overall distribution across two species, and the relatively low nonspecific binding of [^{3}H]LY515300 sites in both rat and guinea pig brain suggests that this radioligand is useful in labeling opioid receptors in native tissues.

Perhaps the most interesting finding in this study was that Na^+ substantially increased high affinity binding of [^{3}H]LY515300 to all three opioid receptors. Moreover, the binding under high Na^+ conditions "unmasked" a population of [^{3}H]LY515300 binding sites that were undetected under low Na^+ conditions. The effect of Na^+ on opioid binding is well documented, increasing antagonist binding affinity while decreasing the affinity of agonists (Pert and Snyder, 1974; Childers and Snyder, 1980; Kosterlitz et al., 1988; Ott and Costa, 1989). Na^+ appears to regulate receptor conformation through interactions with a highly conserved aspartate residue in transmembrane domain 2 of type 1 G protein-coupled receptors (Horstman et al., 1990; Kong et al., 1993; Ceresa and Limbird, 1994). The allosteric modulation of the receptor through this site decreases agonist affinity by promoting an inactive (R_i) receptor confirmation (Zajac and Roques, 1985; Costa et al., 1992). [^{3}H]LY515300 appears to have increased affinity for the inactive (Na^+ bound) state of the receptor, an activity that is consistent with inverse agonist efficacy (Costa et al., 1992; Kenakin, 2001). Similar Na^+-dependent binding has been well documented for the prototypical δ-opioid receptor inverse agonist ICI,174864 (Applemans et al., 1986; Costa and Herz, 1989). Recently, we have reported Na^+-dependent high affinity binding and functional inverse agonist activity at the δ-opioid receptor for LY255582 and other 3,4-dimethyl-4-(3-hydroxyphenyl)piperidines (McKinzie et al., 2001). Given that [^{3}H]LY515300 exhibits Na^+-dependent binding at μ-, δ- and κ-opioid receptors, and that this molecule is a mixture of 3,4-dimethyl-4-(3-hydroxyphenyl) piperidine inverse agonists, suggests that LY515300 and other 3,4-dimethyl-4-(3-hydroxyphenyl)piperidines may have inverse agonist activity at all three classical opioid receptors. These findings are not in opposition to previous studies describing 3,4-dimethyl-4-(3-hydroxyphenyl)piperidines as opioid receptor antagonists, since in an assay system without known constitutive receptor activity, an inverse agonist would be indistinguishable from a neutral antagonist (Kenakin, 2001). Whether the superior efficacy in obesity models of 3,4-dimethyl-4-(3-hydroxyphenyl)piperidines over traditional neutral opioid receptor antagonists such as naltrexone is related to their efficacy as inverse agonists is unknown, but raises an interesting hypothesis for future testing. Further studies with this and other 3,4-dimethyl-4-(3-hydroxyphenyl)piperidines in opioid receptor expression systems with known constitutive activity are presently being conducted to test the inverse agonist hypothesis.

In summary, we have demonstrated that [^{3}H]LY515300 is a high affinity radioligand for analyzing opioid receptors in cell lines expressing recombinant human receptors and in rat and guinea pig brain. In addition, we have shown that high affinity binding of [^{3}H]LY515300 is Na^+-dependent, suggesting that this compound may be the first radiolabeled opioid receptor inverse agonist. Further studies detailing the pharmacology of [^{3}H]LY515300 binding sites in native tissues and directly analyzing inverse agonist activity of 4-phenylpiperidine antagonists are in progress.

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