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Selective opioid receptor agonist and antagonist displacement of [^{3}H]naloxone binding in amphibian brain

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Abstract

Opioid receptor ligands have been shown to elicit antinociception in mammals through three distinct types of receptors designated as μ, δ and κ. These opioid receptors have been characterized and cloned in several mammalian species. Radioligand binding techniques were employed to characterize the sites of opioid action in the amphibian, *Rana pipiens*. Naloxone is a general opioid receptor antagonist which has not been characterized in *R. pipiens*. Kinetic analyses of [^{3}H]naloxone in the amphibian yielded a K_D of 6.84 nM while the experimentally derived K_D value from saturation experiments was found to be 7.11 nM. Density data were also determined from saturation analyses which yielded a B_{max} of 2170 fmol/mg. Additionally, K_i values were calculated in competition studies for various unlabelled μ-, δ- and κ-opioid receptor ligands to isolate their site of action. Highly selective antagonists for μ-, δ- and κ-opioid receptors yielded nearly identical K_i values against [^{3}H]naloxone.

Keywords: Amphibian; [^{3}H]Naloxone; Antinociception; Opioid receptor

1. Introduction

It is widely recognized that opioids produce analgesia in mammalian species through the activation of one or more distinct types of opioid receptors. Evidence for the multiplicity of opioid receptors in mammals originated with behavioral studies (Martin et al., 1976), was strengthened by radioligand binding studies (Gillan et al., 1980; Lord et al., 1977), and was confirmed with identification of genes for three distinct types of opioid receptors (Raynor et al., 1994).

Whereas the multiplicity of opioid receptors in mammals is certain, it is not clear whether opioid actions in non-mammalian vertebrates are mediated by more than one type of opioid receptor. The antinociceptive effects of a number of opioid receptor agonists in amphibians have been well characterized using the acetic acid test (Pezalla, 1983; Stevens and Pezalla, 1983, 1984), which consists of placing a single drop of the lowest concentration of acetic acid on the dorsal surface of a frog's thigh with a Pasteur pipette and then proceeding with increasing concentrations on alternate hind limbs until the nociceptive threshold is reached. The nociceptive threshold is defined as a wiping response directed to the drop of acid using either hindlimb. The antinociception produced by opioid receptor agonists was shown to be opioid receptor mediated as it was significantly blocked by the general opioid receptor antagonists, naloxone and naltrexone (Stevens et al., 1994; Stevens and Pezalla, 1984). Selective μ-, δ-, and κ-opioid receptor agonists elicit consistent and potent antinociception following systemic or central administration (Stevens, 1996; Stevens and Rothe-Skinner, 1997; Stevens et al., 1994). Interestingly, the relative antinociceptive potency of selective μ-, δ- and κ-opioid receptor ligands in amphibians and rodents is highly correlated in both systemic and intraspinal administration studies (Stevens, 1996; Stevens et al., 1994). Based on these findings, radical differences in the receptor proteins between mammals and amphibians would not be expected.

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Previous binding studies in the amphibian have shown predominantly one κ-like opioid receptor binding site with few sites characterized as μ- or δ-opioid receptor binding sites (Benyhe et al., 1990; Simon et al., 1982). It has been determined that this opioid receptor binding site in amphibians is so uniquely different from mammalian opioid receptors that some authors call it as a ''non-μ, non-δ, non-κ''-opioid receptor (Mollereau et al., 1988). No studies thus far have examined a full complement of selective μ-, δ-, and κ-opioid receptor ligands, nor have they used highly selective opioid receptor antagonists in competitive binding assays.

Recently, data from behavioral studies in amphibians employing selective μ-, δ-, and κ-opioid receptor ligands and highly selective opioid receptor antagonists produced a surprising finding: highly selective antagonists for μ- δ-, and κ-opioid receptors were *not* selective in amphibians (Stevens and Newman, 1999). In general, the μ-opioid receptor selective antagonist, β-funaltrexamine, prevented the antinociceptive effects of μ-, δ-, and κ-opioid receptor agonists with the same unexpected finding observed for the δ-opioid receptor selective antagonist, naltrindole, and the κ-opioid receptor selective antagonist, nor-binaltorphimine (Stevens and Newman, 1999). These studies suggest either multiple opioid receptors which binds several types of opioid ligands or a single opioid binding site which mediates antinociception for multiple opioid receptor ligands.

In the present study, a full characterization of [^{3}H]naloxone was performed using kinetic, saturation and competition analyses to provide a pharmacological correlate to behavioral data obtained in *Rana pipiens*. A number of μ-, δ- and κ-selective opioid receptor agonists were competed against naloxone binding. Finally, the highly selective μ-opioid receptor antagonist, β-funaltrexamine (Takemori, 1985), the δ-opioid receptor selective antagonist, naltrindole (Portoghese et al., 1988), and the κ-opioid receptor selective antagonist, nor-binaltorphimine (Takemori and Portoghese, 1992), were assayed against naloxone binding.

2. Materials and methods

2.1. Drugs and radioligand

Drugs used include naltrexone hydrochloride, β-funaltrexamine, morphine and fentanyl obtained from the National Institute on Drug Abuse Drug Supply Program (Mr. Robert Walsh of the Research Technology Branch, Rockville, MD). Dermorphin and [D-Pen^2,D-Pen^5]-enkephalin (DPDPE) were obtained from a commercial source (Bachem Bioscience, Prussia, PA). (5*R*)-(5,4α,7,4α,8,5β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8yl]-4-benzofuranacetamide monohydrochloride (CI977, Enadoline) was obtained from Ms. Carol Germain of Parke-Davis (Ann Arbor, MI). (±)-6-Ethyl-1,2,3,4,5,6-hexahydro-3-[(1-hydroxycyclopropyl)methyl]-11,11-dimethyl-2,6-methano-3-benzazocin-8-ol hydrochloride (bremazocine), 17,17′-bis(Cyclopropylmethyl)-6,6′,7,7′-tetrahydro-4,5,4′,5′-diepoxy-6,6′-(imino)[7,7′-bimorphinan]-3,3′,14,14′-tetrol dihydrochloride (nor-binaltorphimine), [D-Ala^2]-deltorphin-II, dynorphin A-(1-13) and 17-Cyclopropylmethyl-6,7-dehydro-4,5-epoxy-3,14-dihydroxy,7,2′,3′-indolomorphinan hydrochloride (naltrindole) were obtained from Research Biochemicals International (Natick, MA). (+)-4-[(α*R*)-α-((2*S*,5*R*)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide (SNC-80) was obtained from Tocris Cookson (Ballwin,

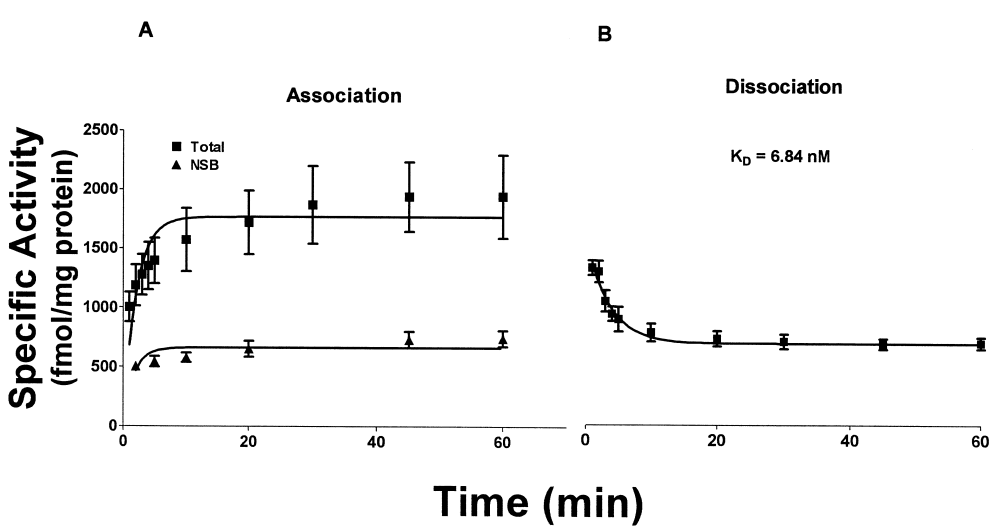

Fig. 1. Association kinetics of [^{3}H]naloxone (5 nM) binding in *R. pipiens* brain (A). Dissociation kinetics in *R. pipiens* brain (B).

Table 1
Kinetically and experimentally derived affinity and density parameters for [^{3}H]naloxone binding

Kinetic analysis		Saturation analysis	
Parameters	Statistics	Parameters	Statistics
$k_{obs}^{a} = 0.4872 \pm 0.2353$	F value = 0.1532, $p = 0.9451$	$K_D = 7.113 \pm 6.37$ nM	F value = 0.1911, $p = 0.8275$
$k_{off}^{a} = 0.2815 \pm 0.1157$	F value = 0.0743, $p = 0.9357$	$B_{max}^{b} = 2170 \pm 600$	–
$k_{on}^{c} = 0.0411$	–		
$K_D^{d} = 6.84$ nM	–		
$B_{max}^{b} = 1767 \pm 211$	–		

[a] min^{-1}.
[b] fmol/mg protein.
[c] $mol^{-1} min^{-1}$.
[d] K_D values were calculated from rate constant on/off values where $K_D = k_{off}/k_{on}$ and $k_{on} = k_{obs} - k_{off}$/[radioligand].

MO). [^{3}H]Naloxone (1.78 TBq/mmol; 48 Ci/mmol) was purchased from Amersham (Arlington Heights, IL).

2.2. Tissue preparation

Crude membrane fractions from untreated frogs were prepared by decapitation followed by rapid excision of the brain. Tissue was stored at −70°C until use in the tissue homogenate binding assay. Brains had a wet weight average of 80 mg. On the day of the experiment, brain tissue was thawed and homogenized in approximately 100 volumes/weight of 50 mM Tris–HCl with 1 mM sodium EDTA, pH 7.4. Pellets were obtained by centrifugation of the homogenate at 400 rpm (29 g) at 4°C for 15 min followed by 14,500 rpm (24,000 × g) at 4°C for 15 min. The resulting pellet was suspended in 50 mM Tris–HCl with 100 mM NaCl, pH 7.4 and rehomogenized for immediate use in the binding assay. Protein analysis was determined according to the Bradford method using bovine serum albumin as the standard (BioRad, Richmond, CA).

2.3. Binding assay

Experiments were performed in triplicate and the receptor binding reactions were initiated by adding [^{3}H]naloxone (50 μl) to 400 μl of tissue homogenate (0.09–0.25 mg of protein) containing either 50 μl of buffer (for total binding) or 50 μl of naltrexone for the determination of

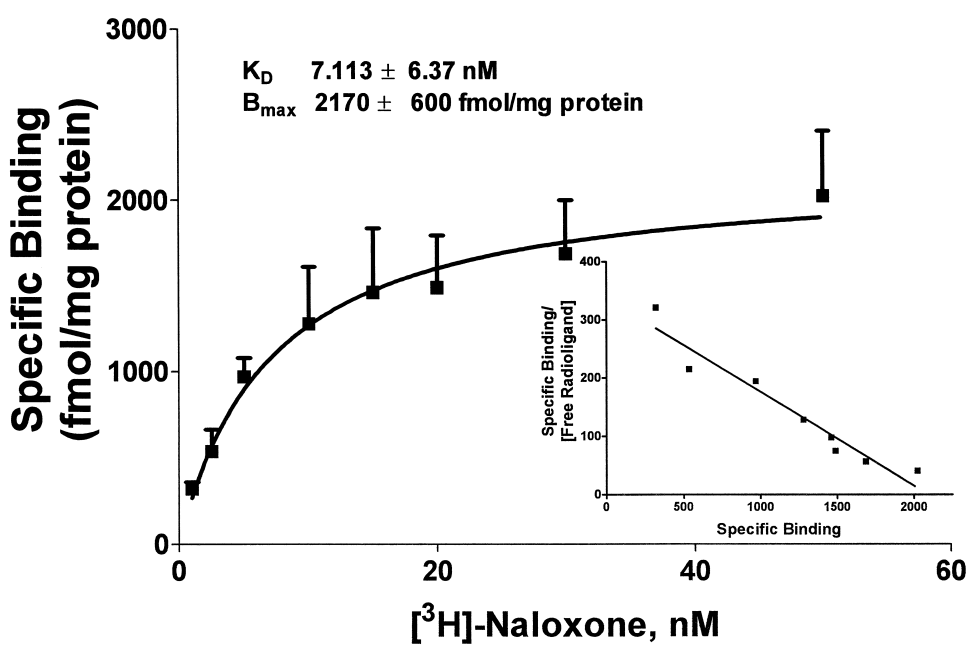

Fig. 2. Saturation analysis of [^{3}H]naloxone in brain tissue homogenates. The membrane preparation was incubated for 60 min at room temperature with various concentrations of [^{3}H]naloxone. Measured binding is the difference between total and nonspecific binding. Values represent the mean of three independent determinations, each performed in triplicate. Inset shows Scatchard analysis of the saturation data. K_D and B_{max} values were determined by the rectangular hyperbole using GraphPad Prism.

nonspecific binding. The components were incubated for 60 min at room temperature in order to equilibrate. Unbound ligand was separated from the receptor–ligand complex and the binding reaction was terminated by rapid filtration using a Brandel 24-cell tissue harvester (Gaithersburg, MD) followed by washing (4×5 ml; 15 s) with cold buffer onto Whatman GF/B glass-fiber filters which were pre-soaked for 1 h in 0.3% polyethylenimine to decrease nonspecific binding. Radioactivity was counted using a Beckman LS1801 scintillation counter (40–50% efficiency) with Scintiverse scintillation fluid (Fisher, Pittsburgh, PA). Specific binding was defined as the difference between non-specific binding (measured in the presence of excess concentrations (10 μM) of naltrexone to block opioid receptor sites) and total binding.

2.4. Kinetic studies

The association component of kinetic analysis involved the addition of radioligand at various time points (10 measurements) where specific binding was measured. Nonspecific binding was defined by a parallel series of tubes containing 10 μM naltrexone and represented 38% of total binding. The dissociation component was accomplished by allowing the radioligand and homogenate to bind to equilibrium at which point further binding was blocked by the addition of 10 μM naltrexone at various time points (10 measurements) where specific binding was measured.

2.5. Saturation studies

Saturation analysis was performed by measuring specific binding over increasing concentrations (0.5–30 nM) of [^{3}H]naloxone to determine receptor density (B_{max}) and affinity (K_D). Nonspecific binding was defined by 10 μM naltrexone. Binding reactions proceeded as described in the binding assay.

2.6. Competition studies

Competition binding experiments were performed using [^{3}H]naloxone with increasing concentrations (15) of unlabelled ligand (0.01 nM–100 μM). 10 μM naloxone was used to define nonspecific binding. Binding reactions proceeded as described in the binding assay.

2.7. Data analysis

Association kinetic analysis involved fitting the data by the one phase exponential association equation or the two phase exponential association equation to determine the best fit. The one phase exponential association equation resulted in the best fit. Dissociation kinetic data were fitted to one and two phase exponential decay to determine the best fit for the data. Like the association data, the one phase equation was the best fit. For saturation analysis the data were first fit to the rectangular hyperbolic function followed by linear transformation (Scatchard, bound/free versus bound). Analysis of the rectangular hyperbola was used to obtain affinity (K_D) and density (B_{max}) data. In competition experiments, concentrations of unlabelled ligand that bind to half of the binding sites at equilibrium (K_i) were calculated by GraphPad using the correction of

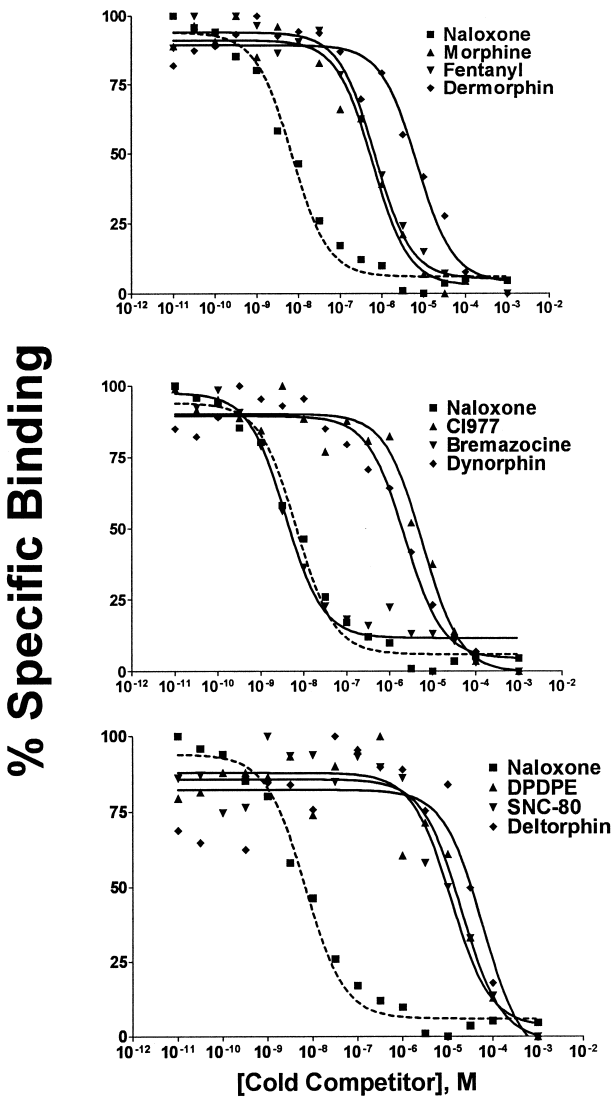

Fig. 3. Inhibition of 5 nM [^{3}H]naloxone binding with various unlabelled opioid receptor ligands in *R. pipiens* brain tissue homogenates. Aliquots of tissue homogenates were incubated for 60 min at room temperature with radioligand in the presence of various concentrations (0.01 nM–100 μM) of cold competitor. Data was normalized to aid comparisons defining the smallest value in the data set as 0% and the largest value as 100% of specific binding. K_i values for these competitors are shown in Table 2. Data points are the means of one representative experiment in triplicate determinations which was repeated three times.

Table 2
Data for the displacement of [^{3}H]naloxone by selective opioid receptor agonists

Drug	Type	K_i (nM)	95% CI[a]	Hill[b]	95% CI
Bremazocine	κ	1.86	(1.12–3.09)	−0.8995	−1.301 to −0.4981
Naloxone	μ,δ,κ	3.92	(2.18–7.03)	−0.9437	−1.436 to −0.4518
Morphine	μ	445	(315–630)	−0.8685	−1.065 to −0.6724
Fentanyl	μ	520	(328–822)	−0.7462	−1.047 to −0.4455
Dynorphin	κ	2381	(1324–4282)	−0.9441	−1.424 to −0.4644
Dermorphin	μ	3025	(1636–5593)	−0.9389	−1.456 to −0.4221
CI977	κ	5124	(1837–14,300)	−1.155	−2.291 to −0.01824
SNC-80	δ	14,140	(7442–26,860)	−0.8403	−1.415 to −0.2659
DPDPE	δ	22,570	(6345–80,280)	−1.068	−2.526 to 0.3906
Deltorphin	δ	31,010	(12,610–76,270)	−1.988	−4.515 to 0.5394

[a]95% Confidence interval.
[b]Hill slope.

Cheng and Prusoff (1973) which corrects for the concentration of radioligand as well as the affinity of the radioligand for its binding site. Competition curves were fitted to one- or two-site binding models, to determine whether the data were best fitted by a one- or two-site model, using the nonlinear least-squares curve-fitting by GraphPad Prism (version 3.00, San Diego, CA) and are based on the statistical *F*-test.

3. Results

3.1. Kinetics of [^{3}H]naloxone binding

Kinetic analysis was performed to determine the time needed to attain the condition of steady-state as well as the rate constants for association and dissociation. Kinetic analyses of [^{3}H]naloxone (5 nM) binding in *R. pipiens* brain homogenates are shown in Fig. 1. Association studies (Fig. 1A) in the brain yielded a k_{obs} (observed association rate) value of 0.4872 min^{-1} while dissociation (Fig. 1B) results yielded a k_{off} (dissociation rate constant) value of 0.2815 min^{-1}. These rate constants yielded a K_D value of 6.84 nM. Statistical analysis of the comparison between one and two site models yielded a best fit for the one site model (see Table 1 for results of *F* test and significance).

3.2. Saturation studies

The properties of naloxone binding sites were studied over an extended range of concentrations of [^{3}H]naloxone where affinity and density data for [^{3}H]naloxone were determined. Saturation data for brain tissue is shown in Fig. 2. As seen in Fig. 2, specific binding was measured over increasing concentrations of [^{3}H]naloxone (0.5–70 nM). Scatchard analysis of these data is shown in the inset. The experimentally derived K_D and B_{max} from saturation analysis were found to be 7.11 nM and 2170 fmol/mg protein, respectively. Kinetic and saturation data for [^{3}H]naloxone are summarized in Table 1. These data were best fit to a one site binding model as determined by the *F* test.

3.3. Competition analysis

In order to clarify drug interaction with particular receptor types, inhibition experiments were performed with selective opioid receptor ligands using [^{3}H]naloxone as the label. Fig. 3 shows these results with Fig. 3A depicting competition with μ agonists, the Fig. 3B showing competition with δ ligands and Fig. 3C, competition with κ receptor agonists. Percent specific binding was measured over a range of concentrations (0.01 nM–100 μM) of cold competitor. For each of these competitors the affinity constant (K_i) was calculated from the complete data set and is shown in Table 2. Additional competition studies with increasing concentrations (0.01 nM–100 μM) of selective antagonists against [^{3}H]naloxone (5 nM) were

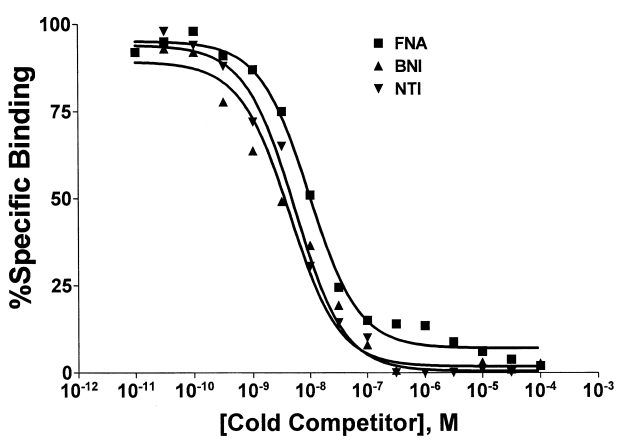

Fig. 4. Displacement of 5 nM [^{3}H]naloxone binding with increasing concentrations (0.01 nM–10 μM) of selective antagonists in *R. pipiens* brain. β-Funaltrexamine is a μ-selective antagonist, naltrindole is δ-selective and nor-binaltorphimine is a κ-selective antagonist. Data was normalized to aid comparisons defining the smallest value in the data set as 0% and the largest value as 100% of specific binding. Data points are the means of one representative experiment in triplicate determinations which was repeated three times.

Table 3
Data for the displacement of [^{3}H]naloxone by selective antagonists

Drug	Type	K_i (nM)[a]	95% CI[b]	Hill[c]	95% CI
β-Funaltrexamine	μ	3.27	2.33 to 4.60	−0.9701	−1.263 to −0.6771
Nor-binaltorphimine	κ	3.55	2.34 to 5.39	−0.7947	−1.094 to −0.4955
Naltrindole	δ	3.22	2.20 to 4.70	−1.021	−1.369 to −0.6739

[a]Apparent K_I value.
[b]95% Confidence interval.
[c]Hill slope.

performed. These results are shown in Fig. 4 with the complete K_i values shown in Table 3. In the case of all competitive ligands, the data were best fit to a one site model as determined by the *F*-test.

4. Discussion

4.1. Opioid action in amphibians

The study of opioid receptor expression in phylogenetically different species has played a significant role in the understanding of opioid receptor pharmacology (Buatti and Pasternak, 1981; Pert et al., 1974). It is well known that three distinct receptors mediate the effects of opioids in mammals. However, previous behavioral studies in *R. pipiens* suggest the possibility of a single opioid receptor, which mediates the actions of μ-, δ- and κ-opioid receptor agonists (Stevens and Newman, 1999). The present results are the first to document the binding characteristics of [^{3}H]naloxone in *R. pipiens* brain homogenates. Additionally, these are the first data to analyze the competition of μ-, δ-, and κ-opioid receptor agonists for naloxone binding sites in *R. pipiens*. Furthermore, the present data are the first to use highly selective opioid receptor antagonists in a competitive binding assay using central nervous system (CNS) tissue from a non-mammalian vertebrate species.

4.2. Naloxone binding affinity and density

Kinetic analysis of [^{3}H]naloxone in *R. pipiens* brain tissue resulted in a kinetically derived K_D value of 6.84 nM. [^{3}H]Naloxone binding was saturable in amphibian brain tissue, yielding a K_D value of 7.11. The experimentally derived K_D value was similar to the K_D value derived from kinetic analysis. This similarity in K_D values, together with the linear transformation of the binding data, is suggestive of binding to a single, noninteractive site but does not rule out binding to several different sites with similar affinity. However, analysis of the data show a best fit to a single site as indicated by the *F* test. Comparable high affinity for [^{3}H]-diprenorphine, a general opioid receptor antagonist, binding to a single site was also seen in *R. pipiens* brain tissue homogenates (Newman et al., 1999). Mammalian studies using brain homogenates show that [^{3}H]naloxone binds to two-sites with affinities ranging from 0.01 to 20 nM (Blurton et al., 1986; Jacobson and Wilkinson, 1984; Pert and Snyder, 1973; Pollack and Wooten, 1987) Density data was also determined in the amphibian where the B_{max} in brain was 2170 fmol/mg protein. This density value in *R. pipiens* as well as values in other amphibian species are higher than those in the mammal where B_{max} values range from 13 to 177 fmol/mg protein (Jacobson and Wilkinson, 1984; Schnittler et al., 1990; Szucs et al., 1987).

4.3. Competitive binding with selective μ-, δ-, and κ-opioid receptor ligands

At mammalian opioid receptor binding sites, naloxone preferentially interacts with expressed μ binding sites (K_D = 3.9 nM, cloned rat receptor) but also has significant affinity for κ-opioid receptors (K_D = 16 nM, cloned rat receptor) and a lesser affinity for δ-opioid receptors (K_D = 95 nM, cloned rat receptor) (Satoh and Minami, 1995). [^{3}H]Naloxone, through competition analysis, has been useful in the determination of receptor affinities of type-selective opioids in mammals (Pfeiffer and Herz, 1982; Schnittler et al., 1990; Szucs et al., 1987). The K_i values for [^{3}H]naloxone binding in frog brain ranged from 1.86 nM for bremazocine to 31,010 nM for deltorphin. As is shown in Table 2, δ-opioid receptor ligands and most κ-opioid receptor drugs were weak displacers of [^{3}H]naloxone binding. The strong displacement of [^{3}H]naloxone by bremazocine is interesting as it is classified as a κ-opioid receptor selective agonist in mammalian studies (Horan et al., 1993), but has been considered a non-selective opioid receptor antagonist in previous binding studies (Broadbear et al., 1994; Wood and Traynor, 1989). This potent displacement of [^{3}H]naloxone by bremazocine was also seen in other amphibian binding studies using [^{3}H]naloxone (Deviche et al., 1990; Simon et al., 1984) as well as in this lab with Chinese hamster ovary (CHO) membrane preparations expressing the human μ-opioid receptors (unpublished data). Additionally, in behavioral studies in this lab, systemically administered bremazocine showed partial agonist/antagonist properties as it significantly blocked the antinociception produced by fentanyl following systemic

administration (data not shown). In examining average K_i values, the overall trend of binding in *R. pipiens* shows a rank affinity of μ → κ → δ-opioid receptor ligands. This affinity profile is consistent with the relative affinity of naloxone for μ-, κ- and δ-opioid receptors (Satoh and Minami, 1995).

4.4. Competition binding with highly selective opioid receptor antagonists

The finding that naloxone bound to a single high-affinity site in amphibian brain and that μ-, δ- and κ-opioid receptor agonists could displace naloxone may be suggestive of a single-type of opioid receptor binding site. To further test this hypothesis, the selective opioid receptor antagonists were used. In mammals these highly selective μ-, δ- and κ-opioid selective antagonists affect the binding of opioid receptor agonists only at their respective receptors (Takemori, 1985; Takemori and Portoghese, 1992). As mentioned above, behavioral studies revealed a lack of selectivity of these antagonists in *R. pipiens*. Interestingly, the three selective antagonists yielded nearly identical K_i values in *R. pipiens* (Fig. 4). Nor-binaltorphimine was examined in another species of frog, *Rana esculenta*, which was shown to have a K_i of 3.1 nM which is nearly identical to that observed in the present studies in *R. pipiens* (Benyhe et al., 1990). K_i values for the selective antagonists in mammals have been determined using selective ligands for the μ-, δ-and κ-opioid cloned receptors. In cell lines expressing the μ-opioid receptor, selective antagonists had K_i values of 0.33 (β-funaltrexamine), 64 (naltrindole) and 2.2 nM (nor-binaltorphimine). At δ-opioid receptors, K_i values were 48 (β-funaltrexamine), 0.02 (naltrindole) and 65 nM (nor-binaltorphimine) and at the κ-opioid receptor, 2.8 (β-funaltrexamine), 66 (naltrindole) and 0.027 nM (nor-binaltorphimine). Thus, each selective antagonist possessed a high affinity binding to its respective receptor and a much lesser affinity for the other two opioid receptors (Raynor et al., 1994). The similar affinities of the selective antagonists in *R. pipiens* in their displacement of [^{3}H]naloxone would suggest that β-funaltrexamine, naltrindole, and nor-binaltorphimine may not bind to separate sites. The present results, together with the previous behavioral data (see above) suggest that opioids may act on a single receptor binding site in amphibians, termed the unireceptor (Stevens and Newman, 1999).

In conclusion, *R. pipiens* represents a unique non-mammalian model for which there is a well-established behavioral assay for testing antinociception produced by opioid receptor ligands. Further studies employing radiolabelled selective agonists are needed to fully characterize the sites of opioid receptor binding in the amphibian. Finally, the ultimate determination of the number and type of distinct opioid receptors in amphibians will come form receptor cloning studies which are currently in progress.

Acknowledgements

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