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Receptor Binding of [^{3}H]Naloxone Benzoylhydrazone: A Reversible κ and Slowly Dissociable μ Opiate

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SUMMARY

In standard ^{3}H-opioid binding assays, the benzoylhydrazone derivative of naloxone (6-desoxy-6-benzoylhydrazido-N-allyl-14-hydroxydihydronormorphinone; NalBzoH) inhibited μ, κ, and δ binding at nanomolar concentrations. At concentrations as low as 1 nM, it also produced a wash-resistant inhibition of opioid binding. [^{3}H]NalBzoH binding typically gave a ratio of total to nonspecific binding of 8:1. Binding reached steady state levels by 1 hr and was linear with tissue concentration. [^{3}H]NalBzoH labeled two classes of sites. The binding to one was easily reversible whereas the other was not and was termed pseudoirreversible. At 25°, almost 90% of [^{3}H]naloxone binding and approximately 60–75% of [^{3}H]NalBzoH binding dissociated over 90 min. However, the remainder of [^{3}H]NalBzoH binding, corresponding to pseudoirreversible binding, remained constant over the next 5 hr at 25° and additional studies suggested a dissociation half-life of approximately 24 hr. Competition studies indicated that the reversible binding corresponded to neither μ nor δ binding and may represent a novel subtype of κ receptor. Pseudoirreversible binding was predominantly to a combination of both μ_1 and μ_2 receptors. Despite its extremely slow rate of dissociation, pseudoirreversible binding was not covalent inasmuch as lowering the pH to 5 or adding the GTP analog 5′-guanylylimidodiphosphate [Gpp(NH)p] completely dissociated prebound [^{3}H]NalBzoH. The ability of Gpp(NH)p to dissociate pseudoirreversible [^{3}H]NalBzoH binding raised the possibility that the slow rate of dissociation was related to interactions with a guanine nucleotide-binding protein.

A variety of novel opiate ligands synthesized over the past few years have proven very useful in the study of opioid action. Of particular importance have been covalent affinity labels or compounds with prolonged durations of action. β-Chlornaltrexamine, one of the first, is not highly selective among the receptor subtypes but has proven useful as a general opiate receptor alkylating agent (1). The selectivity of many of the other agents for μ receptors, such as β-funaltrexamine (2) and 2-(*p*-ethoxybenzyl)-1-diethylaminoethyl-5-isothiocyanobenzimidazole isothiocyanate, or for δ receptors, such as fentanyl isothiocyanate (3) and 14β-(2-bromoacetamido)morphine (4), have provided additional advantages in both biochemical and pharmacological studies. Naloxonazine and naloxazone illustrate another class of opiate ligands (5, 6). Although their mechanisms of action remain uncertain, their prolonged durations of action correlate with a persistent occupation of receptors. Perhaps their greatest advantage is the selectivity of their prolonged receptor blockade for a single subtype of μ receptor, termed μ_1 (7–14, see Ref. 15 for review). Indeed, both compounds have proven very important in correlating different opioid actions with receptor subtypes. For example, μ_1 receptors have been associated with supraspinal analgesia and prolactin release, whereas μ_2 receptors mediate respiratory depression and gastrointestinal transit (see Ref. 15 for review; 16, 17).

Efforts to delineate the mechanism of action of these compounds have led to the synthesis of a number of other compounds that possess similar prolonged durations of action, including diacylhydrazones (18), phenylhydrazones (19), and benzoylhydrazones (20). Most recently, we synthesized the benzoylhydrazone derivative of naloxone, NalBzoH (Fig. 1). We now report the binding of NalBzoH to bovine striatal membranes.

Materials and Methods

All radioligands and Formula 963 scintillation fluor were purchased from New England Nuclear Corp. (Boston, MA). Naloxone HCl was a

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ABBREVIATIONS: NalBzoH, 6-desoxy-6-benzoylhydrazido-*N*-allyl-14-hydroxydihydronormorphinone; DSLET, [D-Ser^2,Leu^5]enkephalin-Thr^6; DADL, [D-Ala^2,D-Leu^5]enkephalin; DAGO, [D-Ala^2,MePh^4,Gly(ol)^5]enkephalin; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; Gpp(NH)p, 5′-guanylylimidodiphosphate; G protein, guanine nucleotide-binding protein; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-proponesulfonate.

generous gift from DuPont (Wilmington, DE) and levallorphan from Hoffman-LaRoche (Nutley, NJ). Morphine sulfate was obtained from National Institute on Drug Abuse Research Technology Branch whereas the opioid peptides were purchased from Peninsula Laboratories (Belmont, CA). Fresh calf brains were obtained locally, dissected into the appropriate brain region, homogenized in 50 volumes of Tris buffer (50 mM, pH 7.6 at 25° with phenylmethylsulfonyl fluoride (0.1 mM), EDTA (1 mM), and NaCl (100 mM), centrifuged (49,000 × *g* for 40 min), resuspended in 0.32 M sucrose, and frozen. Tissue prepared in this manner and kept frozen at −70° retained its binding for at least 3–4 weeks. All binding was performed in potassium phosphate buffer (50 mM; pH 7.2) with $MgSO_4$ (5 mM) for 150 min at 25°, unless otherwise noted, and samples were filtered over Whatman B glass fiber filters using a Brandel Cell Harvester (21–23). For μ_1 assays, we used [^{3}H]DADL (0.7 nM) binding in the presence of DPDPE (10 nM) in 3 ml (15 mg weight of tissue/ml) and, for μ_2 assays, [^{3}H]DAGO (1 nM) binding with DSLET (5 nM) in 3 ml (15 mg wet weight of tissue/ml) of thalamic membrane homogenates. *Delta* binding was measured with [^{3}H]DADL (1 nM) in the presence of morphine (5 nM) in bovine frontal cortical membranes. All δ binding studies were replicated with [^{3}H] DPDPE with identical results. [^{3}H]DPDPE (1 nM) binding was determined in striatal membrane homogenates (2 ml of 10 mg wet weight of tissue/ml). Classical κ binding was determined with [^{3}H]ethylketocyclazocine (1 nM) in the presence of DADL (100 nM) and DAGO (100 nM) to compete against the small amounts of μ and δ binding present in the guinea pig cerebellar membranes (2 ml of 10 mg wet weight of tissue/ml). All determinations were performed in triplicate and replicated three times, unless stated otherwise. Nonspecific binding was determined in the presence of levallorphan (1 μM).

NalBzoH was synthesized using approaches previously reported (19, 20) and was purified by thin layer chromatography. The structure was verified by mass spectroscopy and nuclear magnetic resonance spectroscopy. [^{3}H]NalBzoH (specific activity, 57.4 Ci/mmol) was synthesized, in a similar manner, from [^{3}H]naloxone and was purified on C18 reverse phase SepPak cartridges (Waters, Milford, MA). Its identity was verified by comigration on thin layer chromatography. [^{3}H]NalBzoH (1 nM) binding was performed in bovine striatal membranes (10 mg/ml) in 2 ml of potassium phosphate buffer (50 mM) with $MgSO_4$ (5 mM) at 25° for 60 min, unless otherwise indicated. All assays were performed in triplicate. Nonspecific binding was determined with levallorphan (1 μM) and only specific binding is presented. Each experiment was replicated at least three times, unless otherwise stated. Neither [^{3}H]DAGO nor [^{3}H]NalBzoH binding was altered by benzoylhydrazine at concentrations up to 10 μM, and in some assays unreacted benzoylhydrazine was present at concentrations under 1 μM. To assess pseudoirreversible binding, [^{3}H]NalBzoH was incubated with tissue for 60 min, as described above, and then levallorphan (1 μM) was added and the incubation was continued for an additional 2 hr, to permit the dissociation of reversible ^{3}H-ligand. [^{3}H]NalBzoH binding to filters in the absence of tissue was low, typically 1% or less of filtered radioactivity, and was not changed by including levallorphan (1 μM). Typically, the ratio of total/nonspecific binding ranged between 6:1 to 8:1. In assays designed to measure pseudoirreversible binding, the ratio of total to nonspecific binding was approximately 3:1. Saturation curves were analyzed by regression analysis. Statistical comparisons utilized either analysis of variance or Student's *t* test, depending upon the comparison. Conversion of IC_{50} values to K_i values were calculated as described by Cheng and Prusoff (24) and Chou (25).

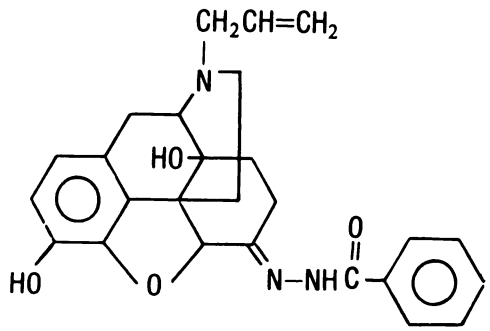

Fig. 1. Structure of NalBzoH.

Results

Opioid receptor selectivity of NalBzoH. First, we examined the selectivity of NalBzoH in several selective binding assays to determine its relative potency against the μ, δ, and κ binding subtypes (Fig. 2). NalBzoH competed against μ_1 binding quite potently and lowered μ_2 binding only slightly less effectively. Against δ binding, NalBzoH was significantly less potent, whereas its activity against κ binding was intermediate. When each of three separate experiments was analyzed independently, the mean K_i values for μ_1, μ_2, κ, and δ binding were 0.3 ± 0.1 nM, 0.8 ± 0.2 nM, 2.3 ± 0.6 nM, and 7.2 ± 0.7 nM, respectively. Thus, NalBzoH competed against all the opioid subtypes tested but was most potent against μ receptors. [Note: the meaning of a K_i value for μ receptors is not clear in view of the [^{3}H]NalBzoH binding presented below.]

Naloxonazine and many of the other hydrazine derivatives of naloxone produce a persistent inhibition of ^{3}H-opioid binding that is not reversed by extensive wash procedures designed to eliminate reversibly bound and free ligand. We therefore determined whether NalBzoH elicited a wash-resistant inhibition of ^{3}H-opioid binding (Table 1). In these studies, tissue was incubated with NalBzoH and then washed extensively to remove freely reversible ligand. After tissue was treated with 1 nM NalBzoH, almost 50% of μ_1 binding was lost. Although slightly less potent against the other subtypes, NalBzoH elicited a dose-dependent inhibition of binding against all radioligands examined.

[^{3}H]NalBzoH binding in bovine brain membranes. First, we established binding parameters for [^{3}H]NalBzoH. Association studies revealed that total specific binding reached equilibrium by 60 min (Fig. 3). Total specific binding was also linear with regard to tissue concentration (Fig. 4). Because binding was linear up to 15 mg wet weight of tissue/ml, we routinely used 10 mg wet weight of tissue/ml in all assays.

Previous studies with the phenylhydrazine derivatives revealed a long, wash-resistant inhibition of binding (16) and we observed a similar inhibition with NalBzoH. We therefore examined the reversibility of [^{3}H]NalBzoH and [^{3}H]naloxone binding at 25° in these membranes (Fig. 5). Total specific [^{3}H] NalBzoH binding was approximately 3-fold greater than [^{3}H] naloxone binding. After the addition of levallorphan (1 μM) to tissue prelabeled with the radioligands, [^{3}H]naloxone rapidly dissociated. Virtually no binding remained after 90 min. In contrast, the dissociation of [^{3}H]NalBzoH binding was biphasic. A portion of binding was readily lost, with a rate similar to that observed with naloxone. However, we typically found that approximately 25–40% of binding was resistant to dissociation, with little change in binding between 2 and 5 hr. Additional studies (data not shown) indicate that this binding persisted for up to 8 hr at 25° and that, by 24 hr, almost 50% of this dissociation-resistant binding still remained. Thus, [^{3}H] NalBzoH labeled two types of sites. Binding to one site, representing approximately 60–75% of binding, was freely reversible, whereas binding to the second site had an extremely slow rate of dissociation and was termed pseudoirreversible because

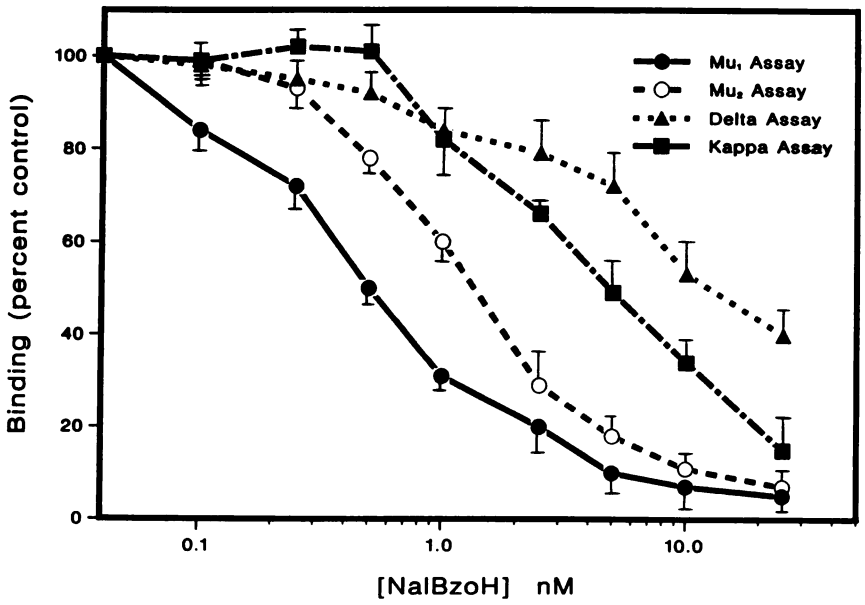

Fig. 2. Competition of ^{3}H-opioid binding by NalBzoH. Competition by various concentrations of NalBzoH in μ_1, μ_2, δ and κ binding assays was determined as described in Materials and Methods. Results represent the means ± standard errors of three independent experiments.

TABLE 1

Wash-resistant inhibition of ^{3}H-opioid binding by NalBzoH

Tissue was treated with NalBzoH at the stated concentration for 30 min at 25°C and then the tissue was washed twice before assays. Tissue treated with naloxone (50 nM) was included in all assays to ensure that the wash was sufficient to remove reversible ligand. In all cases binding in the naloxone-treated tissue was not significantly different from control (untreated) tissue. The results are the means ± standard error of three separate experiments.

NalBzoH	Inhibition		
	μ_1	μ_2	κ
nM		%	
1	46 ± 5	39 ± 5	35 ± 4
5	56 ± 1	43 ± 2	49 ± 4
10	52 ± 2	45 ± 3	53 ± 5
50	60 ± 1	57 ± 3	62 ± 3

it was not covalent (see below). Note that the levels of pseudoirreversible binding were similar to those of [^{3}H]naloxone binding.

Magnesium markedly influenced the levels of pseudoirreversible binding. In the absence of magnesium, pseudoirreversible binding decreased by 60%. EDTA further lowered pseudoirreversible binding; binding was less than 15% of the levels in the presence of magnesium.

The association rate of the pseudoirreversible binding of [^{3}H] NalBzoH was quite similar to that of total specific binding, reaching maximal values within 60 min at 25° (Figure 3), although we occasionally saw a small increase in binding after the first hour. Based upon these results we routinely used one hour incubations. Pseudoirreversible binding was also linear with tissue up to 15 mg/ml (Fig. 4).

We next examined the concentration dependence of [^{3}H] NalBzoH binding (Fig. 6). As the concentration of [^{3}H]NalBzoH increased, total specific and pseudoirreversible binding also increased, reaching maximal binding levels between 3 and 4 nM. Computer analysis of the reversible saturation curve yields an apparent K_D value of 1.1 ± 0.2 nM and a B_{max} of 21 ± 3.5 fmol/mg of tissue (three determinations), whereas the pseudoirreversible binding had an apparent K_D value of 0.7 ± 0.2 nM and a B_{max} of 6.9 ± 0.7 fmol/mg wet weight of tissue (four determinations). However, these estimates must be taken cautiously because the binding may not fulfill the assumptions required by the computer models used to generate the values (26, 27).

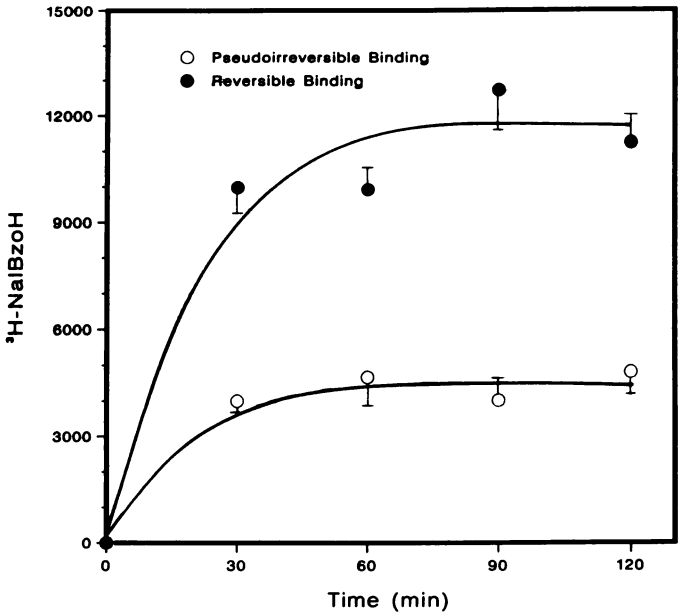

Fig. 3. Association of [^{3}H]NalBzoH binding. Total and nonspecific [^{3}H] NalBzoH binding (1 nM) was determined at 25° at the stated times. Nonspecific binding did not change over the time period examined. Total specific binding was calculated as the difference. Pseudoirreversible binding was determined by incubating the tissue for the stated period of time after which levallorphan (1 μM) was added; the incubation was filtered 2.5 hr later. Reversible binding was determined as the difference between total specific and pseudoirreversible binding. Results are the means ± standard errors of four independent experiments.

A major question regarding the persistent binding was whether it represented a covalent linkage between the ligand and the receptor. We examined this in two ways. First, we studied the effect of lowering the pH on prebound [^{3}H]NalBzoH. ^{3}H-Opioid binding is extremely sensitive to pH and lowering the pH to 5 markedly reduced [^{3}H]naloxone binding

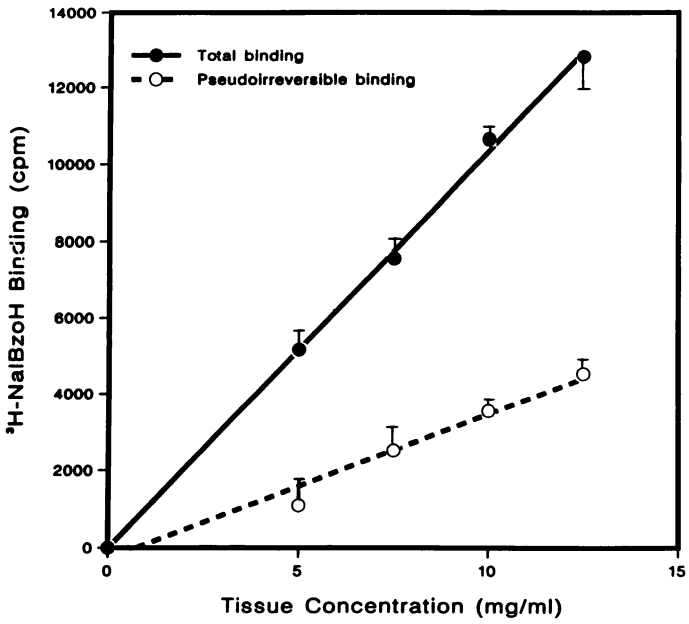

Fig. 4. Tissue linearity of [^{3}H]NalBzoH binding. Bovine striatal tissue, at the stated concentrations, was incubated with [^{3}H]NalBzoH (1 nM) without or with levallorphan (1 μM) for 60 min and filtered to determine total specific binding. Pseudoirreversible binding was determined by incubating tissue with [^{3}H]NalBzoH for 60 min, followed by the addition of levallorphan (1 μM) and filtering 2.5 hr after the levallorphan addition. Results are the means ± standard errors of triplicate determinations from a representative experiment, which has been replicated three times.

(Table 2). [^{3}H]NalBzoH binding was also sensitive to pH 5, implying that the persistent binding was not covalent.

In a second series of studies, we examined the effect of an analog of GTP, Gpp(NH)p, on the dissociation of [^{3}H]NalBzoH binding (Fig. 7). Fig. 5 shows that pseudoirreversible binding remaining after 2.5 hr was very stable for the next 2 hr. In contrast, pseudoirreversible [^{3}H]NalBzoH binding rapidly dissociated in the presence of Gpp(NH)p. Over 85% of binding was lost in the first hour. This sensitivity of [^{3}H]NalBzoH binding to Gpp(NH)p also implied that the binding is not covalent and raised the possibility that pseudoirreversible binding is dependent upon interactions with a G protein.

Selectivity of [^{3}H]NalBzoH binding. In competition studies, NalBzoH inhibited ^{3}H-opioid binding with IC_{50} values in the low nanomolar range. To establish the selectivity of [^{3}H]NalBzoH binding, we performed a series of competition studies (Fig. 8). In all studies, we incubated tissue with the competitor for 2.5 hr before adding the radioligand, to optimize the ability of the reversible agents to inhibit the binding of an irreversible one. Reversible and pseudoirreversible binding were then determined. Morphine added before the [^{3}H]NalBzoH inhibited pseudoirreversible binding quite potently (IC_{50} of 6 ± 1.7 nM, four experiments), but not the reversible binding (IC_{50} of 70 ± 7 nM, four experiments). This 10-fold difference in potency implied that pseudoirreversible binding corresponded to μ sites, whereas reversible binding did not. DAGO, a μ-selective opioid peptide, also competed pseudoirreversible binding quite well (IC_{50} = 6.7 nM, two experiments) but not reversible binding (IC_{50} = 51 nM, two experiments).

DPDPE, a highly selective δ ligand (23, 28), had little effect on either pseudoirreversible or reversible binding, implying that [^{3}H]NalBzoH was not labeling δ receptors. Like DPDPE, DADL labels δ receptors with high affinity but it also binds to μ_1 receptors quite potently, making it a useful tool in discriminating μ receptor subtypes. DADL competed against approximately half of the pseudoirreversible binding, with an IC_{50} of 6.9 ± 2 nM. The remainder of the pseudoirreversible binding was quite insensitive, with an IC_{50} value greater than 100 nM. The sensitivity of pseudoirreversible binding to morphine implied μ receptors. The ability of DADL to compete against approximately 50% of binding suggested that the pseudoirreversible binding represented a combination of both μ_1 (DADL-sensitive) and μ_2 (DADL-insensitive) binding. Competition studies examining DSLET, another enkephalin analog with high affinity for μ_1 and δ receptors, yielded results quite similar to those seen with DADL.

Ethylketocyclazocine, a ligand with high affinity for both μ and κ receptors, inhibited both reversible and pseudoirreversi-

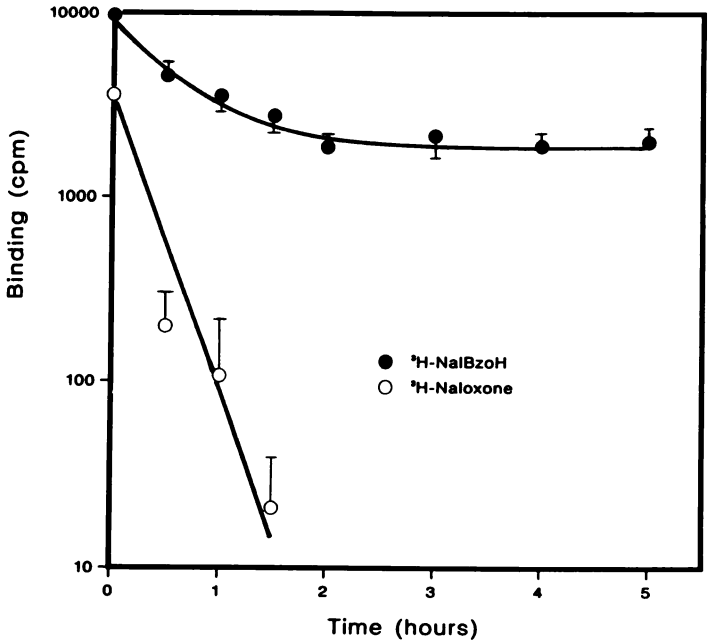

Fig. 5. Dissociation of [^{3}H]naloxone and [^{3}H]NalBzoH binding. Tissue was labeled with either [^{3}H]naloxone (1 nM) or [^{3}H]NalBzoH (1 nM) for 60 min at 25° and then filtered at the stated time after the addition of levallorphan (1 μM). Results are the means ± standard errors of three independent experiments.

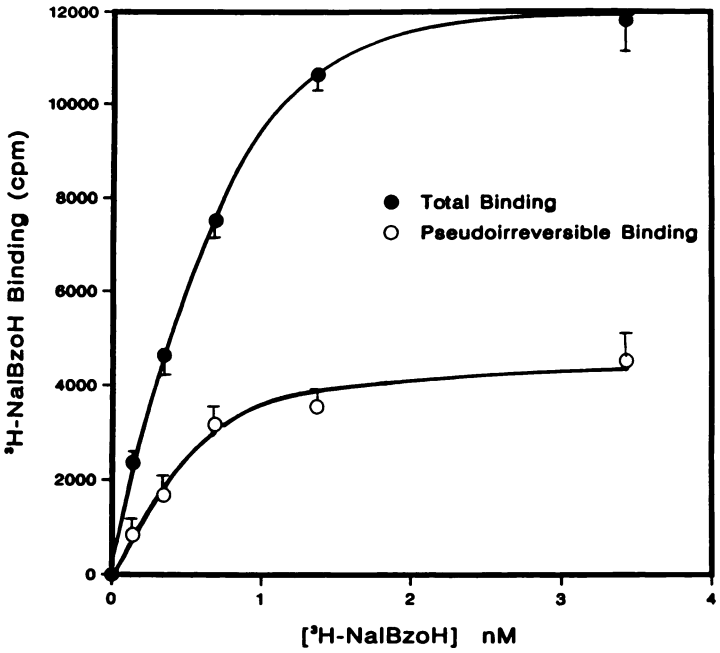

Fig. 6. Saturation study of [³H]NalBzoH binding. Binding was performed with [³H]NalBzoH at the stated concentrations, as described in Materials and Methods. Results are the means ± standard errors of triplicate samples from a representative experiment, which has been replicated three times.

TABLE 2

Effects of low pH on ³H-opioid binding

Control binding was determined at the standard pH (7.2) after a 60-min incubation. Low pH binding represents binding remaining 30 min after the pH was lowered to 5 in tissue prebound with the stated radioligand for 60 min at the standard pH (7.2). Results are the means of triplicate determinations. The experiment has been replicated three times.

	Binding	
	[³H]Naloxone	[³H]NalBzoH
	cpm	
Control	2201 ± 117	9483 ± 178
Low pH	0	0

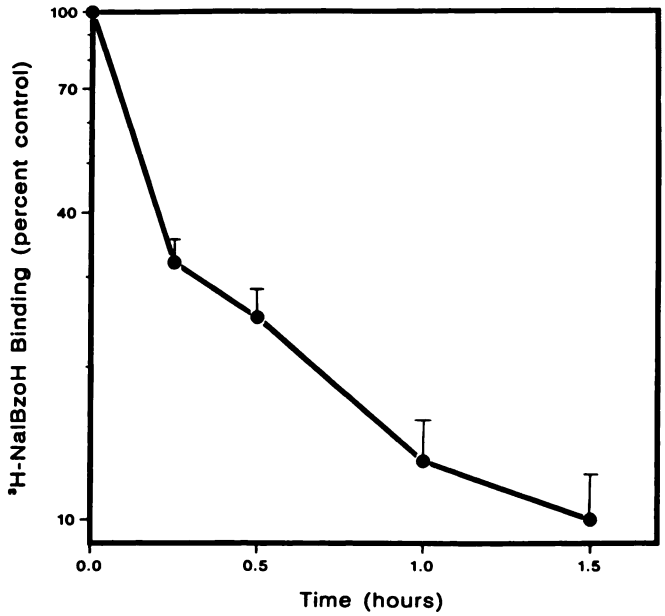

Fig. 7. Effect of Gpp(NH)p on the dissociation of pseudoirreversible [³H]NalBzoH binding. Tissue was bound with [³H]NalBzoH (1 nM), levallorphan (1 μM) was added, and the incubation was extended for an additional 2.5 hr. At this point (time zero), Gpp(NH)p (100 μM) was added and samples were filtered at the stated time after the Gpp(NH)p addition. Results are the means ± standard errors of three independent experiments.

ble binding with similar high potencies. Its effectiveness against pseudoirreversible binding (IC_{50} = 2.4 ± 1.6 nM) was consistent with its similar very high affinity for both μ_1 and μ_2 receptors (K_i = 0.2 nM) (21). The sensitivity to ethylketocyclazocine of reversible binding suggested that this novel binding site might be best classified as a member of κ receptor family.

However, the potent and highly selective κ ligand U50,488 (22, 23) did not appreciably inhibit either reversible or pseudoirreversible binding in the bovine striatum at concentrations up to 100 nM (Fig. 8E). This contrasts markedly with [³H]NalBzoH binding in the guinea pig cerebellum, which contains predominantly classical κ receptors. In the guinea pig cerebellum, U50,488 competed against [³H]NalBzoH quite effectively, with an IC_{50} of 11 ± 2.6 nM. The guinea pig cerebellum data indicate that [³H]NalBzoH can label classical κ receptors, with their documented sensitivity toward U50,488. However, the insensitivity toward U50,488 of the reversible binding in calf striatum demonstrates that this κ-like binding was quite distinct from the classical κ binding sites in the guinea pig cerebellum.

Discussion

NalBzoH has a number of unusual binding properties not observed with classical opiate ligands. Although it inhibits ³H-opioid binding with IC_{50} values similar to those of other opiates, it also produces a wash-resistant inhibition of binding similar to other hydrazone derivatives (5, 18–20). We therefore synthesized [³H]NalBzoH and examined its binding in striatal membranes. The ratio of total to nonspecific binding was quite good, unlike that for [³H]naloxonazine (28), permitting detailed binding studies. We also have examined binding in rat brain, bovine thalamus, and guinea pig cerebellum and, in all cases, the radioligand produced consistent reproducible binding with excellent total to nonspecific ratios, ranging from 8:1 to 4:1, depending upon the tissue.

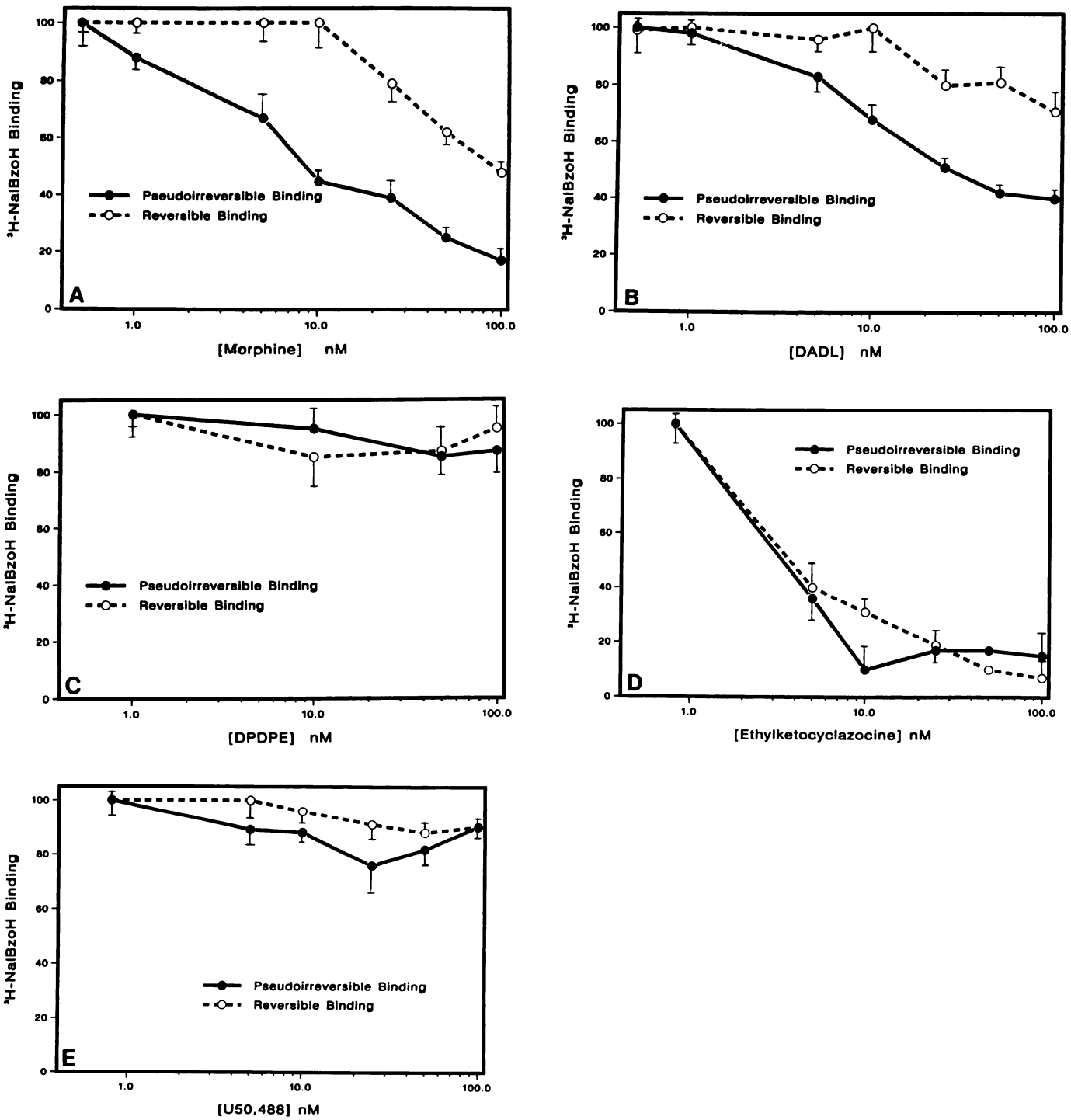

Fig. 8. Competition of [^{3}H]NalBzoH binding. Tissue was incubated with the stated ligand at the designated concentration at 25° for 2.5 hr, then [^{3}H] NalBzoH (1 nM) was added, the incubations were continued, and reversible and pseudoirreversible binding were determined as described in Materials and Methods. Results are the means ± standard errors of three independent experiments and are presented as percentage of control binding. A, Morphine; B, DADL; C, DPDPE; D, ethylketocyclazocine; and E, U50,488.

The reversibility studies of [^{3}H]NalBzoH were quite interesting. As noted in the past with [^{3}H]naloxonazine, approximately 25–40% of total specific binding did not dissociate appreciably over 3 hr at 25° after the addition of high, saturating concentrations of levallorphan (1 μM). By 24 hr, only half of this binding was lost. However, it is not clear whether this represented the dissociation of the ligand from the receptor or simply reflected receptor degradation, which might be expected over this long period. At 0°, the half-life of dissociation increased to 3–4 days. It is interesting to note that total specific [^{3}H]NalBzoH binding was approximately 3-fold greater than [^{3}H]naloxone binding and that the amount of [^{3}H]NalBzoH resistant to dissociation was quite similar to total specific [^{3}H] naloxone binding. In certain respects, the pseudoirreversible binding was anomalous. In the standard ^{3}H-opiate binding competition assays, NalBzoH had IC_{50} values that appeared

similar to those of classical opiates. The concentrations of [3H]NalBzoH required for half-maximal binding also suggested "K_D" values similar to the classical opiates. Yet, the half-life of dissociation was dramatically longer than for classical opioid ligands and could not be explained by a proportionally slower rate of association. In addition to implying a novel binding mechanism, these observations raise serious questions about the meaning of K_D values in this system.

The selectivity of both [3H]NalBzoH binding components was quite interesting. The pseudoirreversible binding was sensitive to morphine and DAGO, implying labeling of μ receptors. DADL also inhibited binding relatively potently, but only approximately 50% of pseudoirreversible binding was sensitive. Because DADL has high affinity for μ_1, but not μ_2, receptors (7), this observation suggested that the pseudoirreversible binding consists of both μ subtypes in approximately equal amounts. DSLET, another peptide with high affinity for μ_1 and δ receptors, gave results almost identical to those observed with DADL. In this regard, [3H]NalBzoH binding is different from pseudoirreversible [3H]naloxonazine binding, which was more μ_1 selective. The B_{max} value for the pseudoirreversible binding, 6.9 ± 0.7 fmol/mg of tissue, agree quite well with the levels of μ_1 and μ_2 binding (21). The classification of pseudoirreversible binding as μ also corresponds quite well to its actions *in vivo*, where NalBzoH is an extremely potent μ antagonist.[1] In mice, NalBzoH reverses morphine (5 mg/kg subcutaneously) analgesia with an ID_{50} of 1 μg/kg, a dose over 5-fold lower than that of naloxone. NalBzoH also reverses morphine lethality and the inhibition by morphine of gastrointestinal transit. In morphine-tolerant mice, NalBzoH rapidly precipitates withdrawal.

The reversible component of [3H]NalBzoH binding was quite unusual. Its density in the bovine striatum was quite high (21 ± 3.5 fmol/mg of tissue), approximately 3-fold greater than the levels of μ receptors. The poor affinity of morphine, DAGO, DADL, DSLET, and DPDPE strongly argue that this binding site does not correspond to either μ or δ receptors. The poor affinity of β-endorphin for this site also argues against an ϵ classification.[2] We feel that the reversible component may represent a previously unrecognized subtype of κ receptor. Evidence supporting this hypothesis includes the high affinity for this reversible component of ethylketocyclazocine and a number of ligands known to be active at κ receptors, including ketocyclazocine, tifluadom, Mr2266, Mr2034, WIN44,441, cyclazocine, and levallorphan.[3] The only ligand with κ activity that did not effectively compete against [3H]NalBzoH binding was U50,488. *Kappa* binding studies have classically utilized tissue from the guinea pig brain, especially the cerebellum (29). In this tissue, U50,488 competes against [3H]NalBzoH binding (IC_{50} = 11 nM) approximately as well as [3H]ethylketocyclazocine (IC_{50} = 12 nM) (22). Recently, several groups have raised the possibility of κ receptor subtypes (30–33). Although U50,488 may be highly selective for κ receptors, it may not be a universal ligand for all members of the κ receptor family. Clearly, the differences in sensitivity to U50,488 between the bovine striatum and the guinea pig cerebellum strongly argue for distinct receptors. The sensitivity of [3H]NalBzoH binding to all the other κ-active agents suggests that it does belong in the κ family of receptors. It is also distinct from the new κ site (κ_2) proposed by Zukin and co-workers (32). Although κ_2 binding is also relatively insensitive to U50,488 (K_i = 484 nM), they report that (−)-ethylketocyclazocine, tifluadom, and cyclazocine compete against κ_2 binding quite poorly (K_i values of 44, 39, and 65 nM, respectively), in marked contrast to reversible [3H]NalBzoH binding, in which all three compounds lowered binding with K_i values under 5 nM.

Despite its extremely slow rate of dissociation, the pseudoirreversible binding was not covalent. Opiate receptor binding is very sensitive to pH changes. Lowering the pH to 5 abolishes the binding of standard 3H-opiates. Similarly, pseudoirreversible [3H]NalBzoH binding dissociated rapidly at pH 5. The ability of Gpp(NH)p to dissociate the pseudoirreversible [3H]NalBzoH binding also argued against covalent bonding and raises a number of questions. Presumably, Gpp(NH)p dissociated [3H]NalBzoH binding through its own interactions with G proteins. The ability of GTP and its analog Gpp(NH)p to modulate receptor binding has been well documented for receptors acting through both stimulatory and inhibitor G proteins (34–42). With other receptor systems, GTP and Gpp(NH)p dramatically reduce agonist binding with little effect on antagonist binding. A similar reduction of opiate agonist binding has also been reported, but, unlike in the other systems, guanine nucleotides inhibit antagonist binding as well (39–42). The marked sensitivity of the pseudoirreversible component of [3H]NalBzoH binding to Gpp(NH)p initially suggested agonist-like binding. However, *in vivo* studies described above indicate that it is a pure μ antagonist, reversing morphine actions and precipitating withdrawal in morphine-dependent mice.

Although the mechanisms involved with pseudoirreversible binding remain speculative, the slow rate of dissociation is unusual and may offer advantages in studies of opiate binding sites. The sensitivity of antagonist binding toward guanine nucleotides is unique among most receptors and further study may yield information about the interactions of μ receptors with G proteins. [3H]NalBzoH remains bound to the receptor after solubilization with CHAPS with the same slow rate of dissociation.[2] This should permit additional studies to characterize the solubilized receptor complex.

In conclusion, NalBzoH is an unusual opiate derivative. In binding assays, it labels μ receptors in a pseudoirreversible manner and recognizes a unique subclass of opioid receptor, which may represent a new distinct class of opioid receptor within the family of κ receptors.

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