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Binding of [^{3}H]Naloxonazine to Rat Brain Membranes

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SUMMARY

[^{3}H]Naloxonazine binds to opioid-binding sites in rat brain homogenates. Prior administration of either morphine or D-Ala^2-D-Leu^5-enkephalin to the homogenates inhibits in a concentration-dependent manner the specific binding of [^{3}H]naloxonazine. Most important, all the binding competed by unlabeled naloxonazine at 1 μM is also competed by morphine and D-Ala^2-D-Leu^5-enkephalin. [^{3}H]Naloxonazine binding is linear with tissue up to 10 mg/ml wet weight of tissue, is temperature dependent, and has a pH maximum of approximately 7.7. Maximal binding is reached within 90 min at 25°. The affinity of [^{3}H]naloxonazine for its binding sites is quite high with half-maximal binding obtained at a concentration of approximately 2 nM. Approximately 40% of the total specific binding of [^{3}H]naloxonazine is resistant to multiple washes and to displacement by levallorphan (1 μM) added 60 min after the [^{3}H]naloxonazine, suggesting that a portion of [^{3}H]naloxonazine binding is not freely reversible. The percentage of total [^{3}H] naloxonazine binding which is not freely reversible varies 3-fold between regions, with the hypothalamus (60%) being the highest and the brainstem (18%) the lowest.

INTRODUCTION

The role of subclasses of opioid-binding sites in the pharmacological actions of the opiates and opioid peptides has become increasingly important in recent years and has depended heavily upon the discovery or development of selective compounds. For example, the interactions of nalorphine with morphine (1) led Martin (2) to postulate two classes of receptors of opiates, a concept he termed receptor dualism. The development of the benzomorphan series of opiates then led Martin to expand his hypothesis to three classes of receptors, which he named after their prototypic drugs: *mu* (morphine), *kappa* (ketocyclazocine), and *sigma* (SKF10,047 or *N*-allylnormetazocine) (3). The discovery of the enkephalins soon led to the realization that specific enkephalin-preferring (*delta*) receptors existed (4, 5). Thus, the discovery and classification of opioid receptor subtypes have relied upon the discovery or development of selective compounds.

All the above receptor classes were determined initially by either *in vivo* or bioassay techniques and later confirmed by receptor-binding approaches. In contrast, evidence initially obtained from binding studies now supports two subtypes of *mu* receptors: mu_1 and mu_2 (6) with the mu_2 site corresponding to the morphine-selective site originally described in the guinea pig ileum (4, 7). The mu_1 site, initially termed "the high affinity site," was first reported (8) soon after initial description of opiate-binding sites and before the isolation and identification of the enkephalins. At first, there was a question of whether this high affinity site might reflect different conformations of a single receptor. The development of naloxazone and naloxonazine (9, 10), two new selective drugs, helped resolve this issue. Both compounds irreversibly and selectively eliminate the high affinity binding component of a number of opiate agonists and antagonists as well as a series of enkephalin derivatives (6, 11–16). Based upon these and other results, Wolozin and Pasternak (6) proposed that morphine and the enkephalins bind to three classes of sites: mu_1 sites which are common high affinity sites ($K_D < 1$ nM) for morphine and the enkephalins; mu_2 sites which preferentially bind morphine (K_D 5–10 nM) and dihydromorphine (K_D 2–4 nM) up to 10-fold more potently than enkephalins like D-Ala^2-D-Leu^5-enkephalin; and *delta* sites which bind enkephalins such as D-Ala^2-D-Leu^5-enkephalin (K_D 5 nM) with over 10-fold greater affinity than morphine.

In vivo studies are consistent with two distinct *mu* receptors. For example, the mu_1-selective antagonist naloxonazine separates the analgesic and respiratory depressant actions of morphine (17). Blockade of mu_1 sites significantly antagonizes morphine analgesia without affecting its respiratory depressant actions, measured with arterial blood samples. Further studies now strongly suggest that respiratory depression is actually mediated

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through mu_2 sites[1]. Similarly, morphine's ability to release prolactin and growth hormone involves separate receptor mechanisms (10).

The actions of naloxonazine have been examined in binding studies (10). Naloxonazine is a selective and irreversible inhibitor of mu_1 sites. Treating rat brain membranes with low concentrations of drug (10–50 nM) produces a concentration-dependent, selective inhibition of mu_1 sites that is resistent to extensive washing, suggesting that the inhibition is irreversible. However, this observation does not necessarily imply covalent mechanism. Indeed, it does not even mean that the drug is still bound to the receptor since the drug could have inactivated the receptor and then dissociated. The washing step is important in the selectivity of naloxonazine for mu_1 sites since naloxonazine will interact reversibly with other sites, such as mu_2. In an effort to further understand the mechanisms involved, we now report the binding of [^{3}H]naloxonazine to rat brain homogenates.

MATERIALS AND METHODS

[^{3}H]Naloxone (48 Ci/mmol), [^{3}H]naloxonazine (77 Ci/mmol; purity > 95% high performance liquid chromatography) and 963 scintillation fluor were obtained from New England Nuclear Corp. (Boston, MA). Male Sprague-Dawley rats (180–220 g) were purchased from Charles River Breeding Laboratories (Wilmington, MA). Unlabeled naloxonazine was synthesized as previously described (10). Naloxone HCl was the generous gift of Endo Laboratories. Glass fiber filters (GF/B) were purchased from Whatman. Trypsin (210 units/mg) was purchased from Worthington Biochemical Corp. (Freehold, NJ). Morphine was obtained from National Institute of Drug Abuse; [D-Ala^2-Leu^5]enkephalin was from Boehringer Mannheim (Indianapolis, IN) and SDS[2] was from Bio-Rad Laboratories (Richmond, CA).

Rat brain membranes were prepared as previously described (18). Centrifugation assays in either potassium phosphate (pH 7.7, 50 mM) or Tris (pH 7.7 at 25°, 50 mM) buffer were performed using either the Microfuge technique or with a high speed refrigerated centrifuge. In the Microfuge technique, all incubations are performed in quadruplicate in plastic Microfuge tubes (1.5-ml tubes with 1 ml of homogenate or 0.5-ml tubes with 0.4 ml of homogenate) in the stated buffer. The tubes are then spun in a Beckman Microfuge for 5 min. A hard pellet is obtained which is then either solubilized in a detergent scintillation fluor or washed by resuspending and centrifuging it again followed by solubilization. In the high speed centrifugation technique, 2-ml aliquots of tissue homogenate are incubated in quadruplicate in 15-ml plastic tubes. The samples are then spun at 49,000 × g for 20 min. At this point, the pellets can either be washed by resuspension with a Polytron and centrifugation or solubilized with the detergent scintillation fluor. All washes include a 20-min incubation at 25° following resuspension to facilitate the dissociation of the drug from its binding site. All binding is reported as specific binding ± standard error of the mean unless otherwise stated. Specific binding is defined as that binding competed for by unlabeled naloxonazine (1 μM). Naloxonazine was used since reversible compounds might not be effective competitors against an irreversible radiolabeled ligand. In assays using [^{3}H]naloxone, the nonspecific binding determined with unlabeled naloxonazine is the same as that obtained with levallorphan (1 μM). Furthermore, all the [^{3}H]naloxonazine binding blocked by the unlabeled naloxonazine can also be competed for by sufficiently high concentrations of morphine. All experiments were replicated a minimum of three times with similar results unless stated otherwise.

Solubilization with SDS was performed by adding 2 ml of 1% SDS solution to pellets from 10 ml of homogenate (100 mg of tissue wet weight) after centrifugation. The pellets were dispersed and the suspension was transferred to a glass tube and immersed in a boiling water bath for 3 min. Protein-bound [^{3}H]naloxonazine was separated from the unbound [^{3}H]ligand by precipitation of the protein with TCA at 10%. The precipitate was solubilized and counted. Protein determinations were performed as previously described (19). Internal standards were also used to ensure that the SDS and TCA did not affect the protein determination. Under these conditions, approximately 65% of the protein is precipitated by the TCA.

RESULTS

Assay techniques for [^{3}H]naloxonazine binding. First we examined [^{3}H]naloxonazine binding using two centrifugation approaches: the Microfuge and the high speed centrifuge. The advantage of the Microfuge technique is its speed and its ability to assay greater numbers of samples. However, the high speed centrifuge permits larger sample volumes and easier resuspension of pellets. Both effectively demonstrate specific binding of [^{3}H] naloxonazine to rat brain homogenates, although the nonspecific binding remains high. Approximately 25–35% of total binding is specific.

Hoping to use the filtration technique, we examined the binding to glass fiber filters of [^{3}H]naloxonazine and [^{3}H]naloxone. The binding to the filters of both ^{3}H-ligands is linear with increasing concentrations of each [^{3}H]ligand. However, the [^{3}H]naloxonazine binding to the filters is approximately 10-fold greater than [^{3}H]

[1] G. S. F. Ling, K. Spiegel, S. H. Lockhart, and G. W. Pasternak, submitted to *J. Pharmacol. Exp. Ther.*

[2] The abbreviations used are: SDS, sodium dodecylsulfate; TCA, trichloroacetic acid.

TABLE 1

[^{3}H]Naloxonazine binding to glass fiber filters

Two-ml aliquots of Tris buffer containing [^{3}H]naloxonazine (67,000 cpm) without (total) and with (displaced) unlabeled naloxonazine (1 μM) were filtered over Whatman GF/B filters previously soaked with the stated compounds 5 min. Results are the means ± standard deviation of triplicate determinations.

Treatment	[^{3}H]Naloxone		[^{3}H]Naloxonazine	
	Total	Displaced	Total	Displaced
Control	825 ± 89	721 ± 21	10,561 ± 1,145	8,079 ± 955
Polyethylenimine (0.1%)	1,032 ± 107	930 ± 30	8,556 ± 553	8,388 ± 423
Naloxone (0.01 mM)	997 ± 18	1,168 ± 43	10,648 ± 638	9,956 ± 925
Naloxonazine (0.01 mM)	850 ± 11	976 ± 191	8,524 ± 406	7,486 ± 488
Albumin (1%)	1,011 ± 91	775 ± 74	6,896 ± 170	7,137 ± 830
Albumin (0.1%)	1,042 ± 67	959 ± 93	8,136 ± 939	8,876 ± 825
Phenelzine (0.1 mM)			11,629 ± 906	

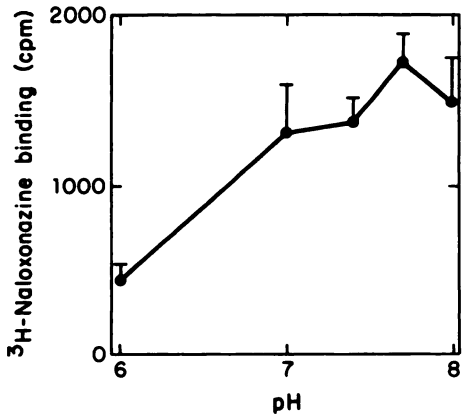

FIG. 1. *pH dependence of [³H]naloxonazine binding*

Rat brain homogenate was suspended in potassium phosphate buffer (50 mM) at the stated pH and incubated in quadruplicate (0.3 ml) at 25° for 90 min in the presence and absence of unlabeled naloxonazine (1 μM) and with [³H]naloxonazine (2 nM). Tissue was pelleted by Microfuge and the pellets were solubilized and counted. Results are the mean ± standard error and represent only displaceable binding. This experiment was replicated three times with similar results.

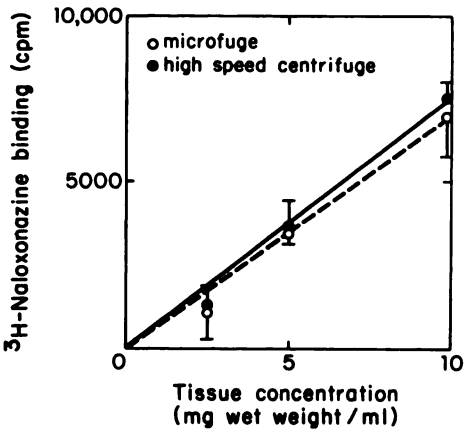

FIG. 2. *Tissue linearity of [³H]naloxonazine binding*

Rat brain homogenate was prepared and diluted with phosphate buffer (50 mM, pH 7.7). Aliquots (2 ml; ●) at the stated concentration of tissue (mg wet weight/ml) in phosphate buffer were then incubated at 25° for 90 min in triplicate in the absence and presence of unlabeled naloxonazine (1 μM) and [³H]naloxonazine (200,000 cpm/tube; 1.2 nM). Samples were then centrifuged (49,000 × *g* × 30 min) and the pellets were solubilized and counted. Results are the mean ± standard error of specific binding from a representative experiment. Alternatively, aliquots (0.3 ml; ○) in Tris buffer were incubated at 25° for 90 min in quadruplicate in the presence and absence of naloxonazine (1 μM) and [³H]naloxonazine (30,000 cpm/0.3 ml; 1.2 nM). Samples were then pelleted by Microfuge (5 min); the pellets were solubilized and counted. Results are the mean ± standard error of specific binding from a representative experiment which have been normalized to a volume of 2 ml. Linearity with tissue concentration up to 10 mg/ml has been replicated four times using the high speed technique and four times using the Microfuge technique (0.3 ml samples).

naloxone at all concentrations of radiolabeled ligand. Efforts to lower this high level of binding to filters with a series of treatments were unsuccessful (Table 1). In addition, some of the [³H]naloxonazine binding to filters is competed for by unlabeled naloxonazine (1 μM). Approximately 2,500 cpm of competable binding are seen with untreated filters. On the basis of these results, we

TABLE 2

Temperature dependence of [³H]naloxonazine binding

Rat brain homogenate (2 ml) was incubated in phosphate buffer at the stated temperature for 90 min in the presence and absence of naloxonazine (1 μM) and [³H]naloxonazine (2 nM). Results are the means ± standard error of specific binding from a representative experiment which has been replicated three times.

Temperature	Binding
	cpm
0°	4,530 ± 1,875
25°	7,361 ± 1,930
37°	11,373 ± 1,672

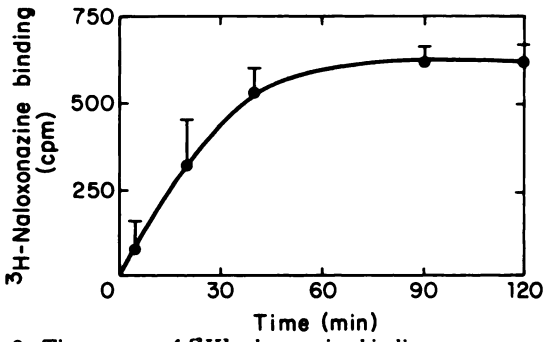

FIG. 3. *Time course of [³H]naloxonazine binding*

Rat brain homogenate (10 mg/ml; 0.3 ml) was incubated in quadruplicate in phosphate buffer in the presence and absence of unlabeled naloxonazine (1 μM) and [³H]naloxonazine (2 nM) at 25° for the stated time. Tissue was pelleted by Microfuge (5 min), solubilized, and counted. Results are the mean ± standard error and represent only specific binding. This experiment was replicated four times with similar results.

concluded that the use of glass fiber filters is not satisfactory for studying [³H]naloxonazine binding and thus routinely used centrifugation assays.

Biochemical properties of [³H]naloxonazine binding. The specific binding of [³H]naloxonazine is pH dependent (Fig. 1), increasing as the pH is raised from 6.0 to 7.7, and linear with tissue concentration only up to 10 mg/ml tissue wet weight (Fig. 2) in both the Microfuge and high speed centrifuge assays. Binding is also temperature dependent (Table 2). Increasing the temperature from 0 to 25° increases binding 60%. Further elevating the temperature to 37° enhances binding an additional 50%. Maximal binding is reached in approximately 90 min at 25° (Fig. 3) and therefore all assays at 25° use a 90-min incubation.

Reversibility of [³H]naloxonazine binding. The inhibition of the high affinity (mu_1) binding of a variety of radiolabeled opiates and opioid peptides following treatment with naloxonazine cannot be reversed by extensive washing (10). We therefore examined the reversibility of [³H]naloxonazine binding (Table 3). Clearly, [³H]naloxonazine binding differs from that of [³H]naloxone. One wash lowers the specific binding of [³H]naloxone over 90% in two separate experiments, while approximately 30–40% of [³H]naloxonazine binding, on the other hand, remains following one to three washes. A single wash also decreases nonspecific [³H]naloxonazine binding by approximately 75%.

The inability of extensive washing to eliminate from

TABLE 3

Effects of washing on [^{3}H]naloxone and [^{3}H]naloxonazine binding to rat brain membranes

Rat brain homogenate was prepared as described in Materials and Methods and 2-ml aliquots in phosphate buffer were incubated at 25° for 90 min in triplicate with either [^{3}H]naloxone (2 nM) or [^{3}H]naloxonazine (2 nM). The homogenate was pelleted at 49,000 × *g* × 30 min. One set of pellets was then solubilized and counted. The other set was then washed, which consisted of resuspension in 10 ml of buffer, incubation at 25° for an additional 20 min, and centrifugation, the stated number of times. Results for [^{3}H]naloxone are reported as two separate experiments (the mean ± standard error of triplicate samples). Results from [^{3}H]naloxonazine are the combined means ± standard error of four separate experiments. Only displaceable binding is reported.

	[^{3}H]Naloxone binding		[^{3}H]Naloxonazine binding
	Experiment 1	Experiment 2	
	cpm		*cpm*
Control	6,217 ± 153	3,779 ± 45	10,638 ± 923
After 1 wash	563 ± 43 (9%)	275 ± 28 (7%)	4,299 ± 1,023 (38 ± 10%)
After 2 washes			3,689 ± 327 (32 ± 2%)
After 3 washes			3,798 ± 754 (36 ± 7%)

TABLE 4

Displacement of [^{3}H]opiate binding by levallorphan

Rat brain homogenate was prepared and incubated with [^{3}H]naloxone (105,000 cpm/2 ml) or [^{3}H]naloxonazine (80,000 cpm/0.3 ml) for 60 min in phosphate buffer in the presence and absence of naloxonazine (1 μM). Either nothing or levallorphan (1 μM) was then added to both sets of 3H-ligands, the incubation was extended for another 30 min, and then [^{3}H]naloxone binding was determined by filtration and [^{3}H] naloxonazine binding was determined by Microfuge. Results are the mean ± standard error of triplicate ([^{3}H]naloxone) or quadruplicate ([^{3}H]naloxonazine) samples from a representative experiment which has been replicated four times.

	[^{3}H]Naloxone binding	[^{3}H]Naloxonazine binding
	cpm	*cpm*
Total specific binding	4,516 ± 446	3,969 ± 398
Nondisplaceable specific binding	161 ± 76 (4%)	1,725 ± 300 (43%)

TABLE 5

TCA precipitation of SDS-solubilized [^{3}H]naloxonazine and [^{3}H] naloxone binding

Rat brain homogenates were prepared (10 mg tissue wet weight/ml) and 10 ml aliquots were incubated with either [^{3}H]naloxone or [^{3}H] naloxonazine (both at 2 nM) for 90 min. Following this incubation, the homogenate was centrifuged for 20 min (49,000 × *g*) and then solubilized by boiling in 2 ml of 1% SDS. One-ml aliquots were then treated with TCA (final 10%) and the precipitate was pelleted, solubilized, and counted. All values are the means ± standard error of four separate experiments. Total and displaced values for [^{3}H]naloxonazine are statistically different ($p < 0.02$) while those of [^{3}H]naloxone are not ($p > 0.5$). Under these conditions, TCA precipitates approximately 65% of the protein.

	TCA-precepitable radioactivity			
	Total	Displaced	Specific	
	cpm			
[^{3}H]Naloxonazine	3,629 ± 322	2,220 ± 339	1,409 ± 328	$p < 0.02$
[^{3}H]Naloxone	488 ± 113	401 ± 112	87 ± 21	$p > 0.5$

30 to 40% of total specific [^{3}H]naloxonazine binding suggests that this wash-resistant binding might not be freely reversible. To test this possibility further, we examined the ability of levallorphan to lower specific binding when added after the ^{3}H-ligands (Table 4). Total specific binding was determined by incubating the homogenate with ^{3}H-ligand for 90 min and then assaying by filtration ([^{3}H]naloxone) or by Microfuge ([^{3}H]naloxonazine). A second set of tissue was incubated with ^{3}H-ligand for 60 min and then levallorphan (1 μM) was added to all tubes. The incubation was continued for an additional 30 min and the samples were assayed. The addition of levallorphan does not significantly affect nonspecific binding. As expected, the addition of levallorphan following the ^{3}H-ligand lowers specific [^{3}H]naloxone binding by over 95%, confirming its reversibility. However, approximately 40% of the specific [^{3}H]naloxonazine binding remains bound even in the presence of excess levallorphan. Thus, once bound, approximately 40% of [^{3}H]naloxonazine binding appears not fully dissociable and resistant to both washing and the subsequent addition of excess opiate.

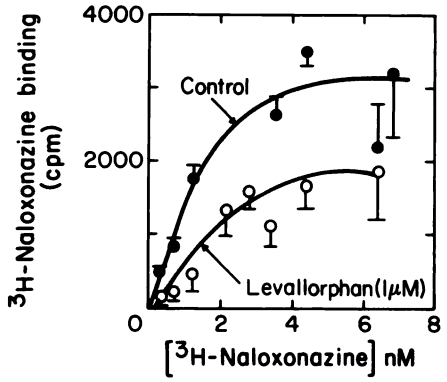

FIG. 4. *Saturation of [^{3}H]naloxonazine binding*

Two sets of aliquots (0.3 ml) of rat brain (10 mg/ml) were incubated in quadruplicate in phosphate buffer at 25° in the presence and absence of naloxonazine (1 μM) and [^{3}H]naloxonazine (0.38 to 6.6 nM). One set was incubated for 90 min. The other set was incubated for 60 min after which levallorphan (1 μM) was added to all the samples and the incubation continued for an additional 30 min. The tissue samples from the control (●) and levallorphan samples (○) were then centrifuged in the Microfuge (5 min); the pellets were solubilized and counted. Results are the mean ± standard error specific binding from a representative experiment which was replicated with similar results three times.

This wash-resistant binding is sensitive to trypsin (250 μg/ml) and *N*-ethylmaleimide (250 μM). Prior treatment of tissue with trypsin followed by centrifugation and

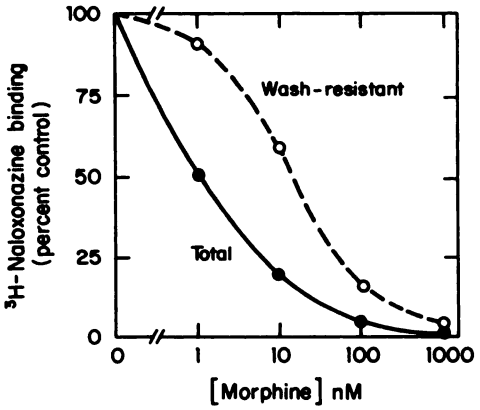

FIG. 5. *Inhibition of [³H]naloxonazine binding by morphine*

Rat brain homogenates (2 ml) were incubated in quadruplicate with morphine sulfate at 25° for 10 min. [³H]Naloxonazine (2 nM) was then added and the incubation continued for 90 min. One series of samples were then centrifuged (49,000 × *g* for 30 min); the pellets were solubilized and counted to determine total binding (●). The other series of samples were washed to determine wash-resistant binding (○). Values are the mean ± standard error of specific binding from a representative experiment. Specific binding was defined as that binding inhibited by naloxonazine (1 μM).

TABLE 6

Regional distribution of [³H]naloxonazine binding in rat brain

In each assay, rat brains ($n = 6$) were dissected, homogenates were prepared (10 mg tissue wet weight/ml), and binding was performed using [³H]naloxonazine (2 nM) and 2-ml aliquots of tissue. Binding was normalized to 10 mg tissue wet weight/ml. Results are the mean ± standard error of n separate binding assays, each performed in quadruplicate.

	Total specific binding	Wash-resistant binding	
	cpm	*cpm*	*% total*
Cortex (frontal) ($n = 2$)	10,322 ± 451	2,773 ± 307	27
Striatum ($n = 2$)	10,987 ± 2,995	3,895 ± 26	35
Hypothalamus ($n = 3$)	3,538 ± 296	2,142 ± 133	60
Brainstem ($n = 2$)	7,725 ± 316	1,404 ± 70	18

resuspension in Tris buffer inhibits wash-resistant binding by 82 ± 18%. Similarly, treatment of tissue with *N*-ethylmaleimide followed by centrifugation and resuspension prior to the binding assay decreases wash-resistant binding 76 ± 12%.

To determine whether the binding which is not freely dissociable might be covalent, we solubilized tissue previously bound with [³H]naloxonazine with the strong denaturing detergent SDS (Table 5). Membranes were incubated with [³H]naloxonazine in the presence or absence of unlabeled naloxonazine (1 μM). The homogenates were centrifuged and the pellets were solubilized in SDS (1% final concentration) in a boiling bath for 3 min. To determine the amount of radioactivity bound to protein under these denaturing conditions, we precipitated the protein with TCA (final concentration, 10%). Protein determinations indicate that approximately 65% of the protein is precipitated with this procedure. Little TCA-precipitable radioactivity is found in tissue bound with [³H]naloxone and none of it is displaced by the inclusion of unlabeled naloxonazine. Quite different results are observed with the [³H]naloxonazine. Approximately 20% of the radioactivity present in the pellet prior to solubilization is TCA-precipitable and almost 40% of this precipitable radioactivity is specific. We also examined the incorporation of radioactivity in tissue samples that had been solubilized with SDS (1%) prior to the addition of the [³H]naloxonazine. There is no specific precipitable radioactivity under these conditions, which is expected, since SDS should destroy all specific binding sites. While there is some radioactivity in the TCA-precipitated pellets, the same amounts are found in samples assayed in the presence and absence of unlabeled naloxonazine (1 μM).

Saturation studies of [³H]naloxonazine binding. We next performed saturation studies on [³H]naloxonazine binding (Fig. 4). Total specific [³H]naloxonazine binding increases with increasing concentrations of free drug, finally reaching a plateau at approximately 4 nM. The concentration of free ligand at which approximately half-maximal binding is observed is between 1 and 2 nM. Similar results are observed in levallorphan-resistant binding. In these studies, levallorphan (1 μM) is added after 60 min and the incubation continued for an additional 30 min. As noted earlier, this procedure permits detection of the extent of reversibility of radioligand binding. The binding remaining after the addition of levallorphan also reaches a plateau at approximately the same concentration of free ligand. Approximately half of the specific binding is not altered by the subsequent addition of levallorphan.

Pharmacological specificity of [³H]naloxonazine binding. Addition of morphine prior to the incubation with [³H]naloxonazine inhibits both total and wash-resistant binding in a concentration-dependent manner (Fig. 5). The concentrations of morphine necessary to inhibit wash-resistant binding are approximately 10-fold greater than those effective against total binding. These results are consistent with the previous findings suggesting that the wash-resistant binding is not dissociable. However, all specific binding can be inhibited by morphine, suggesting that both the wash-resistant and total binding is to opioid-binding sites.

Total [³H]naloxonazine binding is also lowered by [D-Ala²-Leu⁵]enkephalin. In two series of competition studies, morphine (IC_{50} 3 ± 1.6 nM) is approximately 2-fold more potent than the enkephalin (IC_{50} 6 ± 3.2). Although this difference does not achieve statistical significance, it is consistent with the slightly greater *mu* character of naloxonazine (10). Wash-resistant binding is also lowered by [D-Ala²-Leu⁵]enkephalin. At 25 nM, morphine lowers [³H]naloxonazine binding by 49 ± 11% and the enkephalin decreases binding to a similar extent (30 ± 6%; $n = 3$ for both compounds). Again, the ability of both an opioid alkaloid and peptide to completely inhibit all specific binding further emphasizes the opioid nature of [³H]naloxonazine binding.

Regional distribution of [³H]naloxonazine binding. Significant differences exist in the regional distribution of mu_1 (20) and other opioid-binding sites (20–23). Similarly, [³H]naloxonazine binding varies between regions (Table 6). Of the four regions examined, the lowest levels

are in the hypothalamus and the highest levels are in the frontal cortex and striatum. The distribution of wash-resistant binding is quite different. Whereas total specific binding is about the same in cortex and striatum, wash-resistant binding is 40% higher in striatum than in cortex. Total specific binding in the brainstem is over 3-fold greater than in the hypothalamus, while the levels of wash-resistant binding in the two regions are approximately the same. Perhaps the most striking result is the difference in percentage of total specific binding which is wash-resistant in each region. The region with the highest percentage of wash-resistant binding is the hypothalamus (60%) with the lowest being the brainstem (18%). These results correspond to those regions sensitive to naloxazone, a mu_1-selective antagonist (20) and to the regional localization of mu_1 sites using computerized quantitative autoradiography.[3]

DISCUSSION

These studies illustrate a number of features of [^{3}H]naloxonazine binding. In many respects, this binding is quite similar to that of other radiolabeled opiates. Binding is linear with tissue concentration, reaches maximal binding in under 90 min, and has a pH optimum of approximately 7.7. However, there is one significant difference. Approximately 30–40% of total specific binding is not freely reversible. A single wash eliminates over 90% of specific [^{3}H]naloxone binding. Yet, 30–40% of [^{3}H]naloxonazine binding remains following up to three washes. Similarly, less than 10% of [^{3}H]naloxone binding remains following the addition of levallorphan to pre-bound tissue compared to approximately 40% of [^{3}H]naloxonazine binding. The solubilization studies suggest that a portion of this nonfreely reversible binding may be covalent. About 25–30% of the specific wash-resistant binding found in the pellet prior to solubilization is TCA-precipitable and probably reflects binding to specific sites. In view of the strong denaturing properties of SDS, it is very unlikely that the receptor would retain its ability to bind ligands. When membranes are solubilized prior to the addition of [^{3}H]naloxonazine, no specific TCA-precipitable radioactivity is obtained. Thus, the TCA-precipitable radioactivity most likely represents covalently bound drug. Approximately 35% of total protein remains in solution following the addition of TCA and this may explain why only 25–30% of the specific binding is TCA-precipitable. However, a number of additional alternatives are possible.

[^{3}H]Naloxonazine has high affinity for both total and nonfreely reversible binding. Half-maximal binding for both is seen at approximately 1-2 nM ^{3}H-ligand. This affinity of [^{3}H]naloxonazine for the irreversible binding component is greater than expected from previous studies examining the unlabeled compound and may reflect differences in the sensitivities of the two techniques. The earlier studies looked at the effects of naloxonazine on the binding of conventional ^{3}H-ligands, using, in a sense, a negative approach. The actions of naloxonazine are evaluated by determining the loss of binding of reversible compounds. Since the sites sensitive to naloxonazine are few in number, this involves subtracting two relatively large numbers to obtain a small one. The present approach examines the binding directly, permitting a more sensitive and accurate assessment.

The saturation studies also indicate that total [^{3}H]naloxonazine binding corresponds to approximately 12.5 fmol/mg tissue wet weight. This value is quite similar to the total B_{max} values obtained with [^{3}H]naloxone and other classical opiates and opiate peptides. Of this total specific [^{3}H]naloxonazine binding, approximately 5 fmol/mg tissue wet weight is not freely reversible. This value is slightly higher than estimates of mu_1 binding obtained with [^{3}H]naloxone and other ^{3}H-opioids (6, 11–16).

Both the freely and nonfreely reversible specific binding of [^{3}H]naloxonazine appear to correspond to opioid-binding sites. Morphine added prior to the [^{3}H]naloxonazine inhibits total specific binding relatively potently. Prior administration of morphine also inhibits the wash-resistant binding in a dose-dependent manner, implying that this binding represents opioid sites. However, the wash-resistant binding is approximately 10-fold less sensitive, perhaps reflecting the difficulty of inhibiting an irreversible compound with a reversible one. [D-Ala^2-Leu^5]Enkephalin also inhibits total [^{3}H]naloxonazine binding, but with a potency approximately one-half that of morphine. [D-Ala^2-Leu^5]Enkephalin also competes for wash-resistant binding, consistent with the high potency of both morphine and enkephalins at mu_1 sites. Again, the doses of compound needed to displace the wash-resistant binding are higher than those required to compete with conventional ^{3}H-opioids. The most likely explanation is the difficulty in inhibiting the binding of an irreversible ligand with a reversible one.

The studies examining unlabeled naloxonazine demonstrate a selectivity of the irreversible inhibition of binding for mu_1 sites. Our results with the radiolabeled material are consistent with this hypothesis. The wash-resistant binding component is very sensitive to trypsin and *N*-ethylmaleimide and is displaceable by both morphine and [D-Ala^2-Leu^5]enkephalin. This hypothesis is further strengthened by the regional distribution of the wash-resistant binding. Previous investigations examined the sensitivity of [^{3}H][D-Ala^2-Met^5]enkephalinamide binding to naloxazone, another mu_1-selective ligand (20). These experiments demonstrated a regional sensitivity to naloxazone which differed from that of total ^{3}H-opioid binding. Forty per cent of the binding in the hypothalamus is lost following naloxazone treatment while binding in the striatum is lowered 22% and brainstem and frontal cortex are affected hardly at all. Similar regional distributions are seen with the wash-resistant binding. Sixty per cent of [^{3}H]naloxonazine binding in the hypothalamus is wash-resistant, followed by 35% in the striatum. As in the earlier studies with naloxazone, we find the lowest percentages of wash-resistant binding in the frontal cortex and brainstem. It is interesting that the percentage of wash-resistant binding observed with [^{3}H]naloxonazine is generally greater than that determined in the earlier naloxazone experiments. Presumably, these small differences reflect the greater sensitivity

[3] R. R. Goodman and G. W. Pasternak, in preparation.

of measuring the binding directly as opposed to inhibition of reversible ^{3}H-ligands. The greater than 3-fold differences in the percentage of wash-resistant binding between regions strengthens the possibility that the wash-resistant binding reflects a unique receptor population. Furthermore, the distribution of wash-resistant binding corresponds quite well to the localization of mu_1 sites in rat brain determined by quantitative computerized autoradiography.[3]

In conclusion, [^{3}H]naloxonazine appears to label opioid-binding sites. A portion of this binding is not freely reversible and may correspond to mu_1 sites. Further evidence suggests that part of this binding may be covalent. Based upon these results, we feel that [^{3}H] naloxonazine may provide a useful tool in the biochemical characterization of opioid-binding sites.

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