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Opiate Binding in Calf Thalamic Membranes: A Selective μ_1 Binding Assay

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SUMMARY

In the present study, we examined the binding of [^{3}H][D-Ala^2,D-Leu^5]enkephalin ([^{3}H]DADL) to bovine thalamic membranes. Scatchard plots were linear with a K_D of 0.7 nM. However, competition experiments suggested binding heterogeneity. Approximately 20% of [^{3}H]DADL binding was easily inhibited by [D-Pen^2,D-Pen^5]enkephalin (DPDPE) and was insensitive to morphine, implying labeling of δ receptors. The remaining 80% of binding was quite sensitive to both morphine and [D-Ala^2,MePhe^4,Gly(ol)^5]enkephalin (DAGO) and insensitive to DPDPE, consistent with a μ receptor. However, this binding did not correspond to classical morphine-selective μ receptors. Unlike morphine-selective receptors, this binding had similar affinities for morphine, DAGO, DADL and [D-Ser^2,Leu^5]enkephalin-Thr^6 (DSLET). In addition, it was far more sensitive to naloxonazine's wash-resistant inhibition and magnesium-induced enhancement of binding than either the morphine-selective (μ_2) or δ sites. [^{3}H]DSLET binding yielded results very similar to those using [^{3}H]DADL. In conclusion, approximately 80% of [^{3}H]DADL binding in thalamus corresponds to a μ receptor distinct from the classical morphine-selective site. Based upon the results of our studies, we feel that this binding represents μ_1 receptors. DPDPE (10 nM) can effectively inhibit the binding of [^{3}H]DADL to δ receptors, leaving a relatively homogeneous labeling of μ_1 sites. The availability of this selective binding assay should facilitate additional studies of μ_1 receptors.

Soon after the initial demonstration of opiate binding sites (1–3), we observed a very high affinity binding component using both the agonist dihydromorphine and the antagonist naloxone (4). The subsequent identification of the enkephalins (5–8) and of receptors selective for either morphine (μ) or the enkephalins (δ; see Ref. 9) immediately raised questions regarding the relationship of the very high affinity binding site to μ and δ receptors. When we examined this question, evidence from a variety of approaches indicated that this very high binding component did not correspond to either the morphine-selective μ or δ receptors and we proposed that morphine and the enkephalins labeled with high affinity three, not two, classes of binding sites (10–13). In addition to the morphine-selective μ (μ_2) and δ receptors, we determined that morphine and most enkephalins labeled the high affinity site identified in 1975 (4) with similar, very high affinites. Work from several other laboratories also supported this hypothesis (14, 15). Subsequently, sophisticated computer modeling has confirmed this proposal. Self- and cross-competition data were generated for DADL and DAGO and fit using the LIGAND program (16). In brief, Rodbard and Munson and co-workers (17–19) found that the results were best fit by a three-site model consisting of sites selective for either DAGO (μ) or DADL (δ) and a third site present at a lower density, which bound both compounds with very high affinity (μ_1). Similar conclusions were obtained by Toll and Loew and co-workers (20). Although the modeling studies clearly show the presence of a third site, detailed studies of this site have been greatly hampered both by its absence in peripheral tissues and by its low density within the brain.

Pharmacological evidence also supports the concept of multiple μ receptors (for review see Ref. 21). μ_1 Receptors have been implicated in supraspinal analgesia, modulation of acetylcholine and prolactin release, and some aspects of feeding. μ_2 Receptors, on the other hand, mediate different actions, including respiratory depression and inhibition of gastrointestinal transit both at the level of the gut and in the brain (22). If these animals studies can be extended to humans, then μ_1-selective agonists should be effective analgesics without causing respiratory depression and constipation.

Further characterization of μ_1 sites is clearly indicated in view of their potential importance and the development of a simple binding assay would greatly facilitate these studies.

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ABBREVIATIONS: DSLET, [D-Ser^2,Leu^5]enkephalin-Thr^6; DADL, [D-Ala^2,D-Leu^5]enkephalin; DAGO, [D-Ala^2,MePhe^4,Gly(ol)^5]enkephalin; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; U50,488: *trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide.

Typically, at fixed radioligand concentrations, only 25–35% of total specific binding corresponds to μ_1 sites (12, 13, 17–20). The development of an assay, therefore, requires a method of increasing the relative proportion of μ_1 binding while maintaining sufficient levels of binding. We focused upon [^{3}H]DADL binding because of its poor affinity for μ_2 receptors and because of the different regional distribution of δ and μ receptors. Autoradiographic studies of μ_1 binding in rats (23) and mice (24, 25) reveal high levels of μ_1 receptors in the thalamus, with low levels of δ receptors. Almost all the binding of the δ radioligands in this region is inhibited by low morphine concentrations, suggesting that the binding is μ_1. These results suggest that a higher percentage of total specific [^{3}H]DADL binding would correspond to μ_1 receptors. This was confirmed in homogenate binding studies of [^{3}H]DADL in rat thalamus, which demonstrated that greater than 50% of specific binding is inhibited by morphine at 5 nM (26). We now describe ^{3}H-opioid binding to calf thalamic membranes and the development of a μ_1-selective binding assay.

Materials and Methods

Radioligands ([^{3}H]DAGO, [^{3}H]DADL, [^{3}H]DPDPE, [^{3}H]DSLET, and [^{3}H]ethylketocyclazocine) and Formula 963 scintillation fluor were purchased from New England Nuclear Corp. (Boston, MA). 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 phenylmethyl sulfonyl 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. Frozen guinea pig brains were obtained from Charles River (Wilmington, MA). The brains were thawed and the cerebella prepared and frozen as described above.

All binding, with the exception that of [^{3}H]ethylketocyclazocine, was performed in potassium phosphate buffer (50 mM; pH 7.0) with $MgSO_4$ (5 mM) for 150 min at 25° unless otherwise noted and assays were filtered over Whatman B glass fiber filters using a Brandel Cell Harvester. [^{3}H]DADL (0.7 nM) and [^{3}H]DSLET (1 nM) binding was performed using 3 ml (15 mg wet weight of tissue/ml) and [^{3}H]DAGO binding using 3 ml (15 mg wet weight of tissue/ml) of thalamic membrane homogenates. [^{3}H]DPDPE (1 nM) binding was determined in striatal membrane homogenates (2 ml of 10 mg wet weight of tissue/ml). κ Binding was performed with [^{3}H]ethylketocyclazocine in 50 mM Tris buffer (pH 7.6 at 25°). All determinations were performed in triplicate and replicated three times, unless stated otherwise. Nonspecific binding was determined in the presence of levallorphan (1 μM). Only specific binding is reported.

Data analysis. All values are presented as means ± standard errors. Statistical evaluations were determined using either Student's t test or analysis of variance, depending upon the comparisons. K_D and B_{max} values were determined by both nonlinear regression analysis of the saturation data and by linear regression of data transformed according to Scatchard. K_i values of unlabeled compounds were calculated: $K_i = (IC_{50})/(1 + S)$ where S = (concentrations of radioligand)/(K_D of radioligand) (27, 28).

Results

Binding parameters of [^{3}H]DADL binding to thalamic membranes. First, we attempted to characterize the binding of [^{3}H]DADL to the thalamic membranes. Binding was time dependent, requiring 2.5 hr to reach equilibrium at 25° in the presence of $MgSO_4$ (5 mM) (Fig. 1). In the absence of $MgSO_4$, binding approached equilibrium far more rapidly (data not shown). In addition to its effects on rates of association, temperature affected levels of binding at equilibrium. Binding at 37° was approximately the same as at 25°, but the possibility of increased degradation during the prolonged incubation times led us to choose 25° as the assay temperature. Binding at 0° was quite poor, typically less than 20% of binding at 25°. Binding was also influenced by pH, with peak levels around neutral pH that declined rapidly as the pH rose above 8 (Fig. 2). Although binding was similar in potassium phosphate and

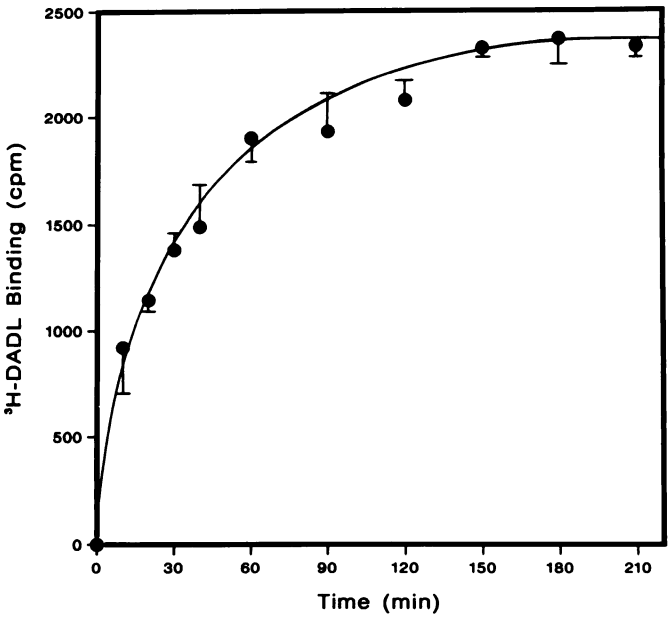

Fig. 1. Time course of [^{3}H]DADL binding. Tissue was incubated with [^{3}H]DADL (0.7 nM) in the presence of $MgSO_4$ for the stated period of time in triplicate with or without levallorphan (1 μM). Results are the means of the triplicate samples, which varied by less than 10%. The experiment has been replicated three times. Only specific binding is reported.

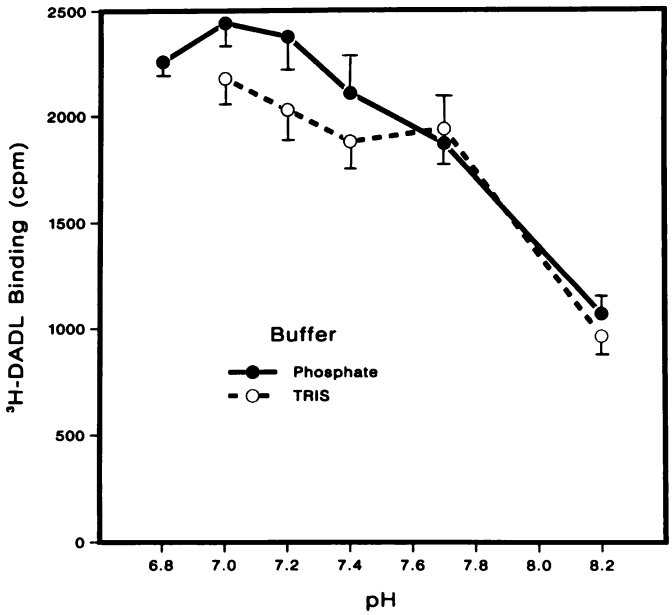

Fig. 2. Effect of pH on [^{3}H]DADL binding. Tissue was incubated with either potassium phosphate buffer (50 mM) or Tris buffer (50 mM) for 150 min and filtered. Results are the means of triplicate determinations. Only specific binding is reported. The assay has been replicated three times.

Tris·HCl buffers at the higher pH values, the phosphate buffer did provide a slight advantage at pH valves around 7. For this reason and for its lack of temperature dependence, we chose to use phosphate buffer routinely.

Because the levels of both ^{3}H-labeled DADL and DSLET binding are relatively low in thalamic membranes, we next examined the relationship of binding to tissue volume and concentration to optimize the binding assay. Binding was linear with increasing tissue concentration to 20 mg/ml wet weight tissue (Fig. 3A). To ensure linearity of binding, we therefore used a tissue concentration of 15 mg/ml in all subsequent studies. With a constant tissue and ligand concentration, binding was also linear with respect to volume up to 3 ml (Fig. 3B) and all subsequent studies used this volume.

Divalent cations modulated [^{3}H]DADL binding in thalamic membranes (Table 1). Although all three divalent cations enhanced binding, magnesium was the most effective. The chelator EDTA decreased binding, as expected. In view of the ability of $MgSO_4$ to increase binding, we routinely incorporated 5 mM $MgSO_4$ in all assays.

Selectivity of [^{3}H]DADL binding in thalamic membranes. Having established the binding parameters, we next performed saturation studies of [^{3}H]DADL binding. Scatchard plots were linear and estimates of K_D and B_{max} values using linear regression for three separate experiments (0.67 ± 0.15 nM and 2.85 ± 0.3 fmol/mg of tissue, respectively) were very similar to values obtained using nonlinear regression analysis (0.71 ± 0.2 nM and 2.89 ± 0.06 fmol/mg of tissue).

Both autoradiographic and binding studies suggested the presence of few δ receptors in the thalamus, raising the question of the selectivity of [^{3}H]DADL binding. Morphine competed with this binding very potently (Fig. 4A). However, a portion of binding remained resistant to inhibition. Nonlinear regression analysis revealed a better fit for a two-site model with IC_{50} values of 1.2 and 100 nM, corresponding to 81 and 19% of total specific binding, respectively. In view of the sensitivity of the majority of binding to morphine, we felt that it most likely represented binding to μ receptors. This was supported by the ability of the μ peptide DAGO, but not the δ-selective DPDPE, to inhibit binding potently (Fig. 4B). Note that approximately 20% of specific binding was effectively blocked by DPDPE at concentrations under 10 nM, similar to the amount of binding resistant to morphine competition. These results suggested that approximately 20% of total specific [^{3}H]DADL binding corresponded to δ sites whereas the remainder represented μ binding. When morphine competitions were performed against [^{3}H]DADL in thalamic membranes with a low dose of DPDPE (10 nM), the morphine-insensitive component was not observed and binding was completely inhibited by morphine at 25 nM (data not shown). Hill coefficients of morphine competitions ranged from 0.65 to 0.8; inclusion of DPDPE (10 nM) in the assay raised the values to between 0.85 and 1.05.

Although the sensitivity of [^{3}H]DADL binding to morphine and DAGO, but not DPDPE, strongly supported the labeling of μ receptors, the high affinity of DADL for this binding site, as determined by saturation studies, raised the question of whether this μ binding corresponded to the classical morphine-selective μ receptor (29, 30). We therefore compared the ability of unlabeled DADL to compete with [^{3}H]DADL and [^{3}H]DAGO binding in thalamic membranes (Fig. 4C). As expected, DADL competed with [^{3}H]DADL binding quite effectively, with an

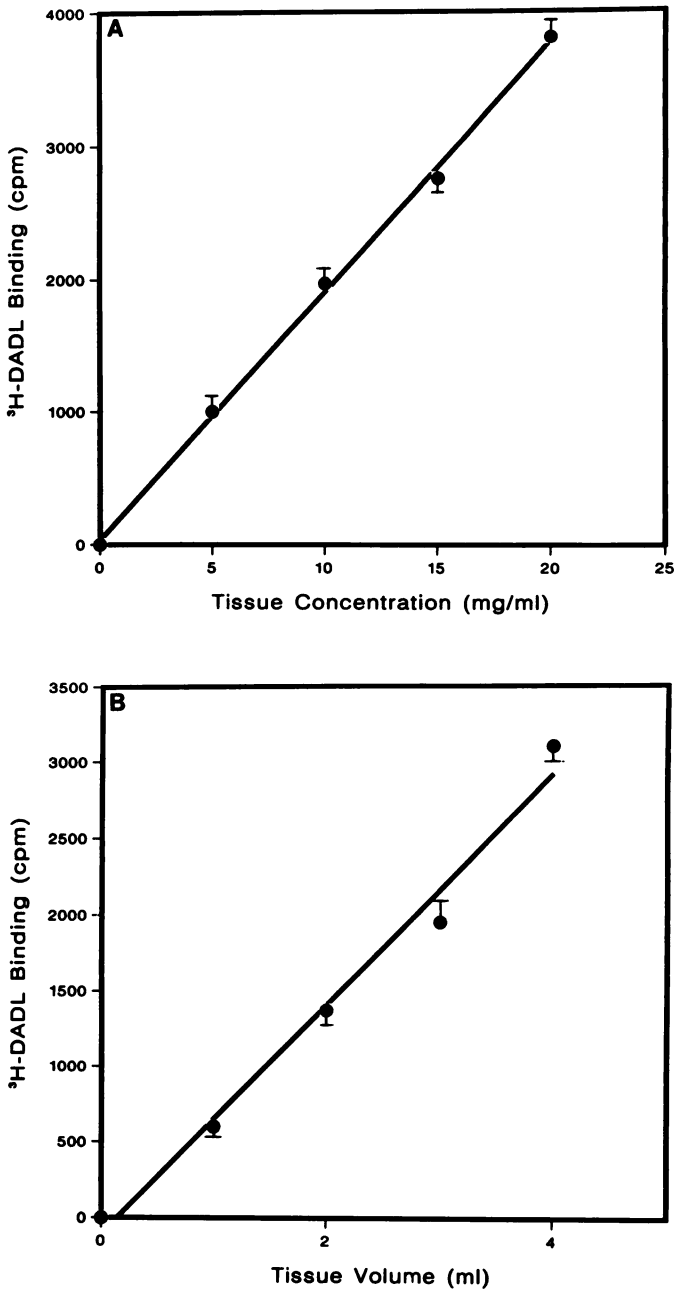

Fig. 3. Relationship of tissue to binding. A, Different concentrations of tissue were incubated with [^{3}H]DADL (0.7 nM) for 150 min in the presence and absence of levallorphan (1 μM). Only specific binding is reported. Results are the means of triplicate samples, which varied by less than 10%. The experiment has been replicated three times. B, Volumes of tissue (15 mg wet weight of tissue/ml) ranging from 1 to 4 ml were incubated for 150 min with [^{3}H]DADL (0.7 nM) in the presence an absence of levallorphan (1 μM). Only specific binding is reported. Results are the means of triplicate samples, which varied by less than 10%. The experiment has been replicated three times.

IC_{50} value of approximately 1–2 nM, consistent with its K_D value of 0.7 nM observed in saturation studies. However, DADL was much less effective against [^{3}H]DAGO binding. Similar results were observed with DSLET (Table 2). Although it potently lowered [^{3}H]DADL binding, it was almost 5-fold less potent against [^{3}H]DAGO binding. Unlike DADL and DSLET, morphine lowered both [^{3}H]DADL and [^{3}H]DAGO binding equally potently, with an IC_{50} around 2 nM, implying that both

TABLE 1

Effects of cations on [^{3}H]DADL binding in thalamic homogenates

Tissue was prepared and assays performed with [^{3}H]-DADL in the absence or presence of the stated compound (1 mM). Binding is expressed as a percentage of control and represents the results of three separate assays, each of which was performed in triplicate.

Addition	Change in binding
	%
$CaCl_2$	+34 ± 3
$MgSO_4$	+61 ± 4
$MnCl_2$	+43 ± 7
K_2EDTA	−35 ± 8

radioligands were labeling μ receptors. However, the differences in potency of DADL and DSLET against the two radioligands strongly suggested μ receptor heterogeneity.

Having demonstrated the high potency of morphine in competing with [^{3}H]DADL binding, we next examined the nature of the competition. If both morphine and DADL were labeling the same site, their inhibition should be competitive. Inclusion of a low concentration of morphine (1 nM) in saturation studies (Fig. 5) revealed no significant change in B_{max} and a prominent change in apparent affinity, consistent with competitive interactions.

Inclusion of sodium chloride (100 mM) in the assay lowered [^{3}H]DADL binding by 40–50%. Saturation studies of [^{3}H]DADL binding indicated a decrease in affinity with little change in binding capacity (data not shown). To determine whether the binding remaining in the presence of sodium chloride retained μ_1 characteristics, we examined the ability of several compounds to inhibit binding (Table 3). In all cases, the IC_{50} values of competitors increased, but their relative potency did not change appreciably. Both μ and δ ligand still lowered binding with similar potencies, with the exception of DPDPE. Thus, sodium chloride decreased ligand affinity without changing the competition pattern from that of a μ_1 site to that of either an enkephalin-selective (δ) or morphine-selective (μ_2) binding site.

DSLET binding to thalamic membranes. DSLET is another widely used δ peptide that labels both μ_1 and δ sites (31). Because it has a receptor selectivity similar to that of DADL, it should yield results similar to DADL. Saturation studies of [^{3}H]DSLET binding reveal linear Scatchard plots with a K_D value of 1.6 nM. Morphine inhibits [^{3}H]DSLET binding competitively, as determined by Scatchard analysis. In competition studies, morphine inhibited [^{3}H]DSLET binding (1 nM) with an IC_{50} value of approximately 1 nM. Thus, the binding of DSLET corresponds quite well to that of DADL.

Comparison of μ_1, μ_2, and δ binding. The results of the above studies strongly suggested that the majority of [^{3}H]DADL binding to thalamic membranes corresponded to μ_1 binding. Competition studies implied that about 20% of total specific binding was to δ receptors and that DPDPE at 10 nM would effectively compete with this binding. Thus, [^{3}H]DADL binding in the presence of DPDPE should provide a selective μ_1 binding assay. Using this assay, we next compared the affinity of a series of opiates and opioid peptides for μ_1, μ_2, δ and κ binding sites (Table 4). δ Binding was determined with the highly selective ligand [^{3}H]DPDPE in the striatum. μ_2 Binding was determined in the thalamus using [^{3}H]DAGO in the presence of DSLET (5 nM). κ Binding was determined in the guinea pig cerebellum using [^{3}H]ethylketocyclazocine in the presence of 100 nM DADL and 100 nM DAGO to block μ and δ binding. These assay conditions yield reasonable estimates of affinities of the compounds for the various receptor subtypes.

As expected from earlier studies, all the opiates and opioid peptides tested had greatest affinity for μ_1 sites, with the exception of DPDPE and U50,488, which were highly selective for δ and κ receptors, respectively, relative to both μ subtypes. The selectivity of the compounds in μ_2 and δ assays were similar to previous reports in the literature for binding to morphine-selective μ and δ binding sites. Finally, all three sites were highly stereoselective. Neither (+)-naloxone nor (+)-levallorphan lowered binding appreciably at 1 μM.

The selectivity of some of the endogenous peptides was interesting, particularly the dynorphins. Dynorphin A(1-17) and (1-13) both labeled μ receptors more potently than δ but were most active against κ binding. Removing amino acid residues at the N-terminus to yield dynorphin A(10-17) markedly lowered affinity at κ sites, over 100-fold, while only decreasing affinities at μ and δ receptors approximately 10-fold. Compared with dynorphin A(1-17), dynorphin A(1-8) was more potent against δ receptors but approximately 10-fold less potent against μ binding and 100-fold less effective against κ sites. It is interesting that dynorphin A(1-8) and (10-17) had about the same activity against μ receptors even though they had no amino acid sequence overlap. This might suggest that the μ receptor recognition site is large, with both ends of the peptide playing an important role. In contrast, the N-terminal portion appears to be far more important to δ binding than the COOH-terminus. One difficulty in interpreting these results, however, is the possibility that degradation may differ from one brain region to another. Although this would not affect the comparison between μ_1 and μ_2 assays that use the same tissue, values against δ and κ binding might be affected. We included some protease inhibitors in an effort to minimize this problem, but it still remains.

The binding selectivity of dynorphin B differed from that of dynorphin A. Although it retained a similar potency at δ receptors, it labeled μ_1 and μ_2 sites approximately 10-fold less effectively. However, its competition in the κ binding assay was most interesting. The Hill coefficient against total specific binding was approximately 0.5 and detailed competition studies elicited a biphasic inhibition of binding, which was best described with two IC_{50} values. The other ligands with high affinity for κ sites, including U50,488, ethylketocyclazocine, and dynorphin A, competed with binding in a monophasic manner.

Naloxonazine is a relatively selective, long lasting μ_1 antagonist both *in vitro* and *in vivo* (13, 21, 32, 33). We examined the sensitivity of μ_1, μ_2, and δ binding to naloxonazine treatment using the selective assays (Fig. 6). μ_1 Binding was clearly more sensitive to naloxonazine treatment than either of the other subtypes. Naloxonazine at 25 nM inhibited greater than 50% of μ_1 binding.

Earlier, we pointed out the ability of magnesium sulfate to elevate μ_1 binding. In more detailed experiments, magnesium increased binding almost 3-fold at 5 mM and plateaued at 10 mM (Fig. 7). Although magnesium also increased μ_2 and δ binding, these effects were far less prominent than the enhancement of μ_1 binding.

Finally, we studied the sensitivity of binding to *N*-ethylmaleimide (Table 5) and trypsin (Fig. 8). *N*-Ethylmaleimide low-

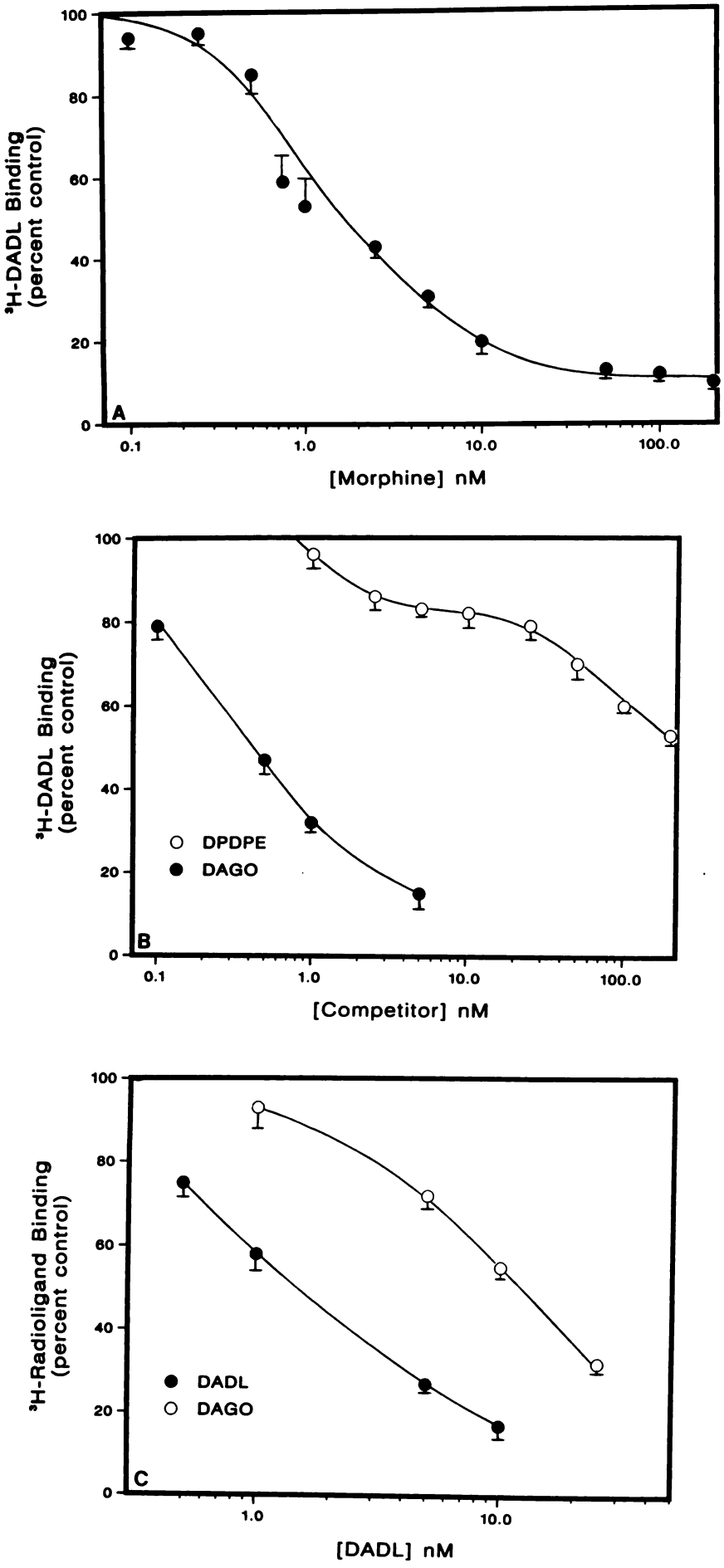

Fig. 4. Competition of [^{3}H]DADL and [^{3}H]DAGO binding in thalamic membranes. A, [^{3}H]DADL binding (0.7 nM) was determined in the presence of varying concentrations of morphine. B, [^{3}H]DADL binding (0.7 nM) was determined in the presence of varying concentrations of DAGO and DPDPE. C, [^{3}H]DADL and [^{3}H]DAGO binding was determined in the presence of varying concentrations of unlabeled DADL. In all figures, nonspecific binding was determined with levallorphan (1 μM). Only specific binding is reported. Results are means ± standard errors of triplicate samples. All experiments have been replicated three times.

TABLE 2

Opioid competition of [^{3}H]DAGO and [^{3}H]DADL binding

Competitions against [^{3}H]DAGO and [^{3}H]DADL (both 0.7 nM) by morphine and DSLET were performed three times and the IC_{50} values were determined. Results are the means ± standard errors of three separate experiments. The IC_{50} values for morphine against the two radioligands were not statistically significant from each other or from DSLET against [^{3}H]DADL. The DSLET IC_{50} value for [^{3}H]DAGO was highly significantly ($p < 0.001$) different from either DSLET against [^{3}H]DADL or morphine against [^{3}H]DAGO.

	IC_{50}		Ratio
	[^{3}H]DADL	[^{3}H]DAGO	
	nM		
Morphine	1.9 ± 0.6	2.7 ± 0.5	1.4
DSLET	3.0 ± 0.8	13.7 ± 0.7	4.6

ered μ_1 binding most effectively. μ_2 Binding was also relatively sensitive, but δ binding was far less affected (Table 4). Trypsin also lowered the binding in the three assays in a concentration-dependent manner. Again, μ_1 binding was most sensitive.

Discussion

The primary purpose of this study was to develop a selective binding assay for μ_1 receptors. By restricting ourselves to the calf thalamus for a source of membranes, we have now developed a simple straightforward μ_1 binding assay. Optimizing the binding was not difficult. We basically increased both tissue concentrations and volume and included $MgSO_4$. However, we were surprised at the slow rate of association in the presence of magnesium, considering that in its absence equilibrium was attained in under 1 hr. More detailed studies have raised the possibility that this slow rate of association may be related to a relatively slow interaction between the receptor and a guanine nucleotide-binding protein.[1] Dissociation rates of binding in the presence of $MgSO_4$ are slow as well, with a half-life of greater than 2 hr at 25°. Thus, the inclusion of magnesium enhances binding but requires extended incubations. This may be a problem with competitors subject to chemical or enzymatic degradation. When dealing with those, binding assays without magnesium using 45–60-min incubations may be more suitable.

[1] J. A. Clark and G. W. Pasternak, manuscript in preparation.

In thalamic membranes, [^{3}H]DADL labeled two classes of sites. Approximately 20% of specific binding corresponded to δ receptors. It was easily inhibited by the δ-selective ligand DPDPE and was relatively insensitive to morphine. Inclusion of DPDPE in the binding assay eliminated the presence of the morphine-insensitive binding, confirming that the 20% of binding observed with the two competitors alone corresponded to the same site. The Hill coefficients seen with morphine alone were relatively low, consistent with binding to more than one site, and inclusion of DPDPE (10 nM) raised it to approximately 1. In view of these results, we felt that the inclusion of DPDPE (10 nM) in the assays would give a relatively homogeneous labeling of μ_1 binding sites.

Clearly, the remaining 80% of binding did not correspond to either the classical μ or δ sites. The sensitivity of this remaining binding to both morphine and DAGO, two highly μ-selective opioids, strongly implied binding to a μ receptor. Its insensitivity towards the highly selective δ ligand DPDPE supported its classification as μ. However, several characteristics differentiated this binding from the classical morphine-selective (μ) binding sites originally reported. First, [^{3}H]DADL labeled these sites quite potently in saturation studies with an affinity (K_D, 0.7 nM) similar to that of morphine (K_i, 0.5 nM and DSLET (K_D, 1.6 nM), distinguishing it from the morphine-selective (μ_2) sites. Morphine inhibited binding competitively, consistent with a common binding site, and 5-fold less potently than its selective (μ_2) site.

The sensitivity of the [^{3}H]DADL binding observed in the thalamus towards both morphine and DAGO strongly argues for the labeling of μ receptors, but this binding differed from that of the μ ligand [^{3}H]DAGO. Although morphine competed with the binding of both ligands with very similar IC_{50} values, both DADL and DSLET were less potent against [^{3}H]DAGO binding than against [^{3}H]DADL binding. [^{3}H]DADL binding displayed a selectivity previously reported for μ_1 receptors. Both μ and most δ ligands competed binding with very high affinity. [^{3}H]DAGO binding, on the other hand, demonstrated the selectivity of μ over δ ligands previously reported for the classical morphine-selective μ receptor. We observed this selectivity because under these conditions [^{3}H]DAGO labeled predomi-

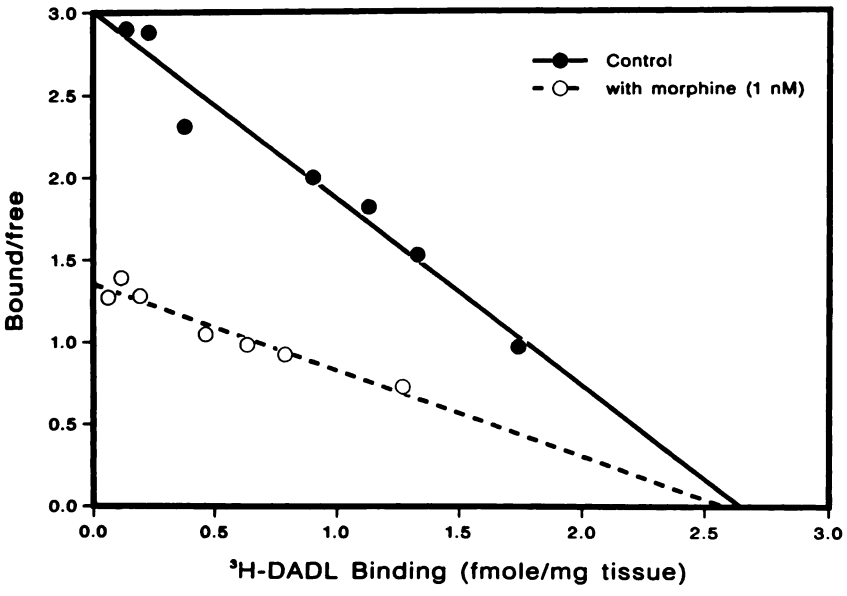

Fig. 5. Scatchard analysis of [^{3}H]DADL binding alone and in the presence of morphine. [^{3}H]DADL binding (0.04 to 1.7 nM) was determined in triplicate in the presence and absence of morphine (1 nM). Additional triplicate samples were assayed in the presence of levallorphan (1 μM) to establish nonspecific binding. K_D values in the absence and presence of morphine were 0.66 and 1.4 nM, respectively. The study has been replicated three times with similar results.

TABLE 3

Inhibition of [^{3}H]DADL binding by opioids in the presence of sodium chloride

Binding was determined using [^{3}H]DADL (0.7 nM) and thalamic membranes in the presence of NaCl (100 mM). IC_{50} values were determined by probit analysis. Each of two experiments was assayed in triplicate and the results are the means ± standard errors.

	IC_{50}
	nM
Morphine	6.9 ± 0.6
DADL	3.4 ± 0.1
DSLET	5.2 ± 0.2
DPDPE	298 ± 33

nantly μ_2 sites. Although the affinity of [^{3}H]DAGO for μ_1 sites is higher than for μ_2, this difference is overshadowed by the far greater numbers of μ_2 sites.

One issue that has received extensive investigation is the ability of sodium ions to decrease the affinity of opiate agonists (34). We also found that [^{3}H]DADL and [^{3}H]DSLET binding was sensitive to sodium ions in a concentration-dependent manner. In view of the suggestion by some that sodium can convert one class of receptor to another, we felt that we had to examine this issue directly. Inclusion of sodium increased the IC_{50} values for all the ligands we examined but did not alter the selectivity of binding. Again, the sensitivity of binding to morphine and not DPDPE strongly argued that the binding was μ whereas the similar potencies of morphine, DADL, and DSLET ruled out the morphine-selective μ receptor. Thus, sodium alters the affinity of opioids to μ_1 sites without converting them to either morphine-selective μ or δ receptors.

Comparison of binding affinities of a series of opiates and opioid peptides in selective binding assays revealed values similar to those estimated from computer analysis of rat brain homogenates. Most of the ligands labeled μ_1 sites with highest affinity. DPDPE was an important exception, confirming previous observations (35). Although it bound to δ sites quite potently, with a K_i of 3 nM, it had a 30-fold lower affinity towards μ_1 sites. U50,488 is another exception (36). This κ-selective ligand competes κ binding 30-fold more effectively than μ_1 binding. Other ligands showed high affinity for both μ_1 sites and either μ_2 (ethylketocyclazocine) or δ sites (DADL and DSLET). Some of the compounds had higher K_i values for μ_1 sites than we had anticipated from rat studies. Several possi-

TABLE 4

Inhibition of ^{3}H-opioid binding by opiates and opioid peptides

Each of the competitors was tested using at least three concentrations within an assay and each assay was replicated at least three times. K_i values were calculated as described in Materials and Methods. Values for U50,488 were obtained from Clark and Pasternak (36). To optimize the μ_1 assay, we used [^{3}H]DADL (0.7 nM) in the presence of DPDPE (10 nM) in thalamic membranes. DPDPE at 10 nM concentration is on the plateau region of its competition curve against [^{3}H]DADL binding and calculations indicate that it should inhibit approximately 75% of δ [^{3}H]DADL binding, leaving about 5% of total specific binding corresponding to δ sites. DPDPE at 10 nM should compete less than 10% of μ_1 binding. μ_2 binding used [^{3}H]DAGO (0.7 nM) in thalamic membranes in the presence of DSLET (5 nM). DSLET at 5 nM should lower μ_1 binding by 55–60% with only a 15% inhibition of μ_2 binding. Because μ_1 binding usually corresponds to only 25–30% of total specific binding, less than 15% of total specific binding should correspond to μ_1 sites. δ Binding used [^{3}H]DPDPE (1 nM) in striatal membranes and κ binding used [^{3}H]ethylketocyclazocine (1.4 nM) in guinea pig cerebellular membranes with DAGO and DADL (100 nM each) as noted in Materials and Methods. All assays examining the peptides included leupeptin (5 μg/ml) and bacitracin (10 μg/ml). In full competition studies, dynorphin B inhibited [^{3}H]ethylketocyclazocine binding in a biphasic manner with a Hill coefficient of 0.5. Values reported above are replicate determinations of the two components of the competition curves. Numbers in parentheses, number of replications.

Competitor	K_i			
	μ_1	μ_2	δ	κ
	nM			
μ				
Morphine	0.50 ± 0.38 (8)	2.5 ± 0.6	278 ± 49	
DAGO	0.50 ± 0.68 (4)	2.1 ± 0.8	>500	
Morphiceptin	55. ± 35	131 ± 48	>500.	
Diprenorphine	0.2 ± 0.2	0.5 ± 0.3	1.4 ± 0.6	
PL-017[a]	5.4 ± 1.6	16.5 ± 1.8	>100	
κ				
Ethylketocyclazocine	0.17 ± 0.06	0.24 ± 0.18	4.7 ± 0.5	2.2 ± 0.1
U50,488	370 ± 76	>500	>500	12 ± 2.4
δ				
DADL	0.9 ± 0.83	7.2 ± 3.1	1.9 ± 0.2	
DPDPE	82. ± 19	457. ± 149 (4)	2.9 ± 0.7	
DSLET	1.4 ± 0.82	14. ± 6.5	2.3 ± 0.8	
Antagonists				
(−)-Naloxone	1.3 ± 0.5	3.7 ± 0.7	106. ± 23	
(+)-Naloxone	>1000	>1000	>1000	
(−)-Levallorphan	0.25 ± 0.17	1.0 ± 0.2	5.4 ± 0.8	
(+)-Levallorphan	>1000	>1000	>1000	
Peptides				
β-Endorphin	0.98 ± 0.14	3.1 ± 0.34	2.6 ± 0.46	>500
α-Neoendorphin	5.6 ± 1.5	19 ± 7	4.2 ± 1.4	12 ± 1.3
Dynorphin A				
(1-17)	0.69 ± 0.16	2.2 ± 0.38	8.7 ± 1.5	0.37 ± 0.1
(1-13)	0.48 ± 0.13	1.5 ± 0.1	8.2 ± 1.9	0.42 ± 0.1
(1-8)	11 ± 3.5	53 ± 10	4.1 ± 1.1	>500
(10-17)	8.2 ± 2	21 ± 1.7	51 ± 18	>500
Dynorphin B	6.0 ± 1.6	17 ± 4.4	6.8 ± 1.3	0.2, 1.5 31, 29

[a] Tyr-Pro-MePhe-D-Pro-NH_2.

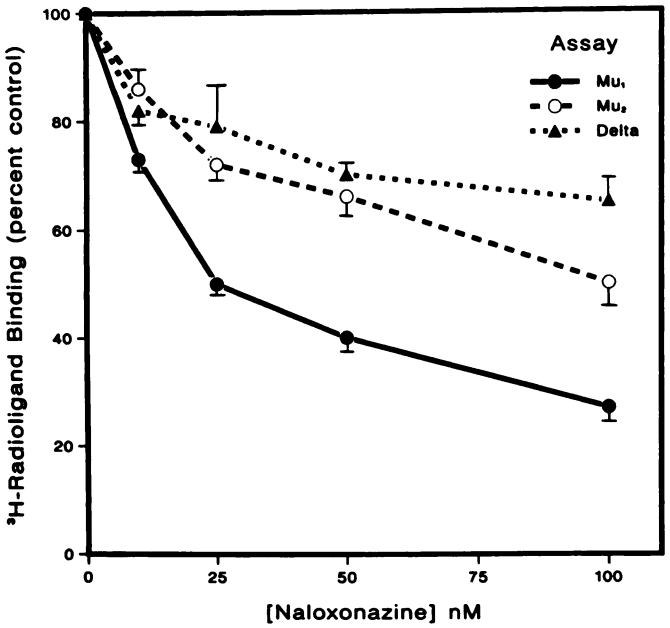

Fig. 6. Sensitivity of ^{3}H-opioid binding to naloxonazine. Tissue was treated with naloxonazine at the stated concentration for 30 min at 25° and then washed twice by centrifugation and resuspension. Control tissue was incubated with naloxone (100 nM) to ensure removal of free and reversibly bound ligand. μ_1 and μ_2 binding was determined in thalamic membranes using [^{3}H]DADL with DPDPE (10 nM) or [^{3}H]DAGO (0.7 nM) in the presence of DSLET (5 nM), respectively. δ Binding was determined using [^{3}H]DPDPE (1 nM) as described in the Materials and Methods. Results are the means of three separate assays. The values for μ_1 binding differed from those of the other two assays at naloxonazine concentrations of 25 ($p < 0.05$), 50 ($p < 0.005$), and 100 ($p < 0.005$) nM, as determined by analysis of variance.

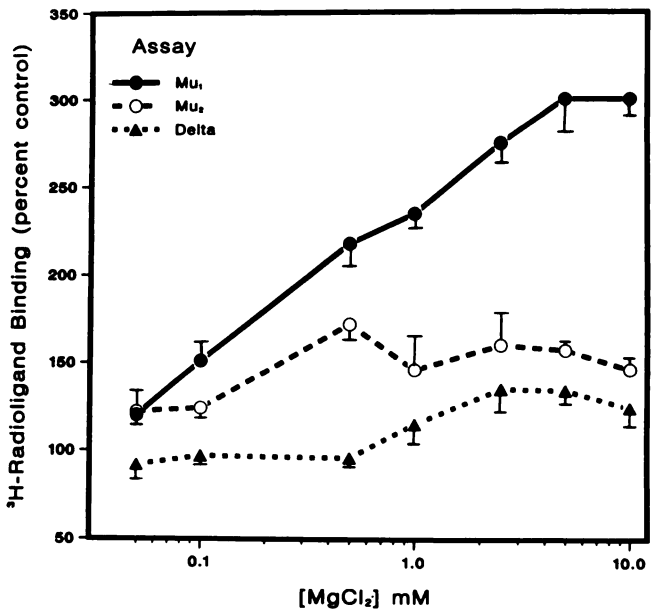

Fig. 7. Sensitivity of ^{3}H-opioid binding to magnesium chloride. Binding was performed in the absence or presence of various concentrations of magnesium chloride. μ_1 and μ_2 binding was determined in thalamic membranes using [^{3}H]DADL with DPDPE (10 nM) or [^{3}H]DAGO (0.7 nM) in the presence of DSLET (5 nM), respectively. δ Binding was determined using [^{3}H]DPDPE (1 nM) described in the *Materials and Methods*. Results are expressed as percentage of control values based upon the means of triplicate samples. The study has been replicated three times. μ_1 Binding was significantly more sensitive to magnesium ($p < 0.05$) than either μ_2 or δ.

bilities might be considered. The current studies used frozen instead of freshly prepared tissue, included magnesium in the assay, and required extended incubation times. In addition, some species differences may exist between calf and rat.

The selectivity of some of the endogenous peptides was interesting, particularly the dynorphins. β-Endorphin and α-neoendorphin possessed reasonably high affinity for μ and δ receptors, but only α-neoendorphin competed κ binding. The high affinity of dynorphin A(1-13) and (1-17) for κ receptors is consistent with previous reports, but their high potency for μ receptors makes them a candidate for the endogenous μ ligand as well. Dynorphin A(10-17) illustrates the differences between μ and κ binding sites. Removal of the first nine amino acids decreased μ and δ binding by approximately 10-fold, in contrast to the several hundred-fold loss in κ affinity. These findings imply that the N-terminal amino acids are very important for κ activity. The ability of dynorphin A(10-17) to compete μ binding as potently as dynorphin A(1-8), even though the former lacks the standard N-terminal opioid pentapeptide sequence and the two peptides have no sequence overlap, suggests that the μ binding site is large and recognizes an extended peptide structure in contrast to δ receptors, which bind the extended derivatives less effectively. Clearly, these structure-activity relationships need to be examined in greater detail and the question of selective degradation of the peptides in the different binding assays needs to be addressed as well.

The dynorphin B competition studies were quite revealing. Unlike all the other ligands, dynorphin B competed [^{3}H]ethylketocyclazocine binding in a biphasic manner, consistent with

TABLE 5

Effect of *N*-ethylmaleimide on ^{3}H-opioid binding

Tissue was assayed as described in Materials and Methods after treatment with the *N*-ethylmaleimide at the stated concentration for 30 min at 37°, centrifugation, and resuspension. Results are the means ± standard errors of three separate experiments, each of which was assayed in triplicate. μ_1 binding was significantly more sensitive to *N*-ethylmaleimide (25 and 50 μM) than the others ($p < 0.05$).

N-Ethylmaleimide	Inhibition of binding		
	μ_1	μ_2	δ
μM		%	
10	7 ± 4	12 ± 8	16 ± 4
25	61 ± 5	46 ± 2	29 ± 12
50	63 ± 2	52 ± 3	30 ± 13

binding heterogeneity. The sensitivity of both sites to U50,488, along with the blockade of μ and δ sites, strongly implies that this heterogeneity represents κ receptor subtypes that can be discriminated by dynorphin B. In the other assays, dynorphin B retained approximately the same affinity for δ receptors as dynorphin A but was about 10-fold weaker against μ receptors.

Naloxonazine selectively antagonizes μ_1 receptors in a long lasting manner (13, 32, 33). As anticipated, μ_1 binding was significantly more sensitive to prior exposure to naloxonazine than either μ_2 or δ. *In vivo* studies with naloxonazine suggested that its selectively is highly dependent upon the dose of drug (37). We observed similar results in the selective binding studies. First, naloxonazine lowered μ_2 and δ binding, but to a much smaller extent than μ_1 sites. Our results also point out that total blockade of μ_1 sites would almost certainly be associated with a significant loss of binding to other sites as well. Naloxonazine remains a useful tool in examining μ_1 receptors, but

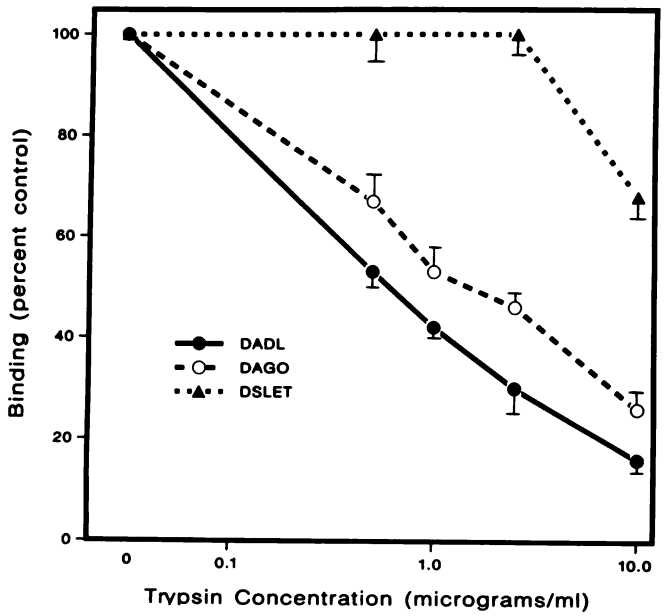

Fig. 8. Sensitivity of ^{3}H-opioid binding to trypsin. Tissue homogenates were incubated without or with trypsin at the stated concentration for 30 min at 25°, after which soy bean trypsin inhibitor was added a concentration 4-fold that of trypsin (weight to weight). The tissue was then centrifuged at 49,000 × *g* for 20 min, resuspended, and assayed. Binding was performed as previously described. [^{3}H]DADL with DPDPE (10 nM) and [^{3}H]DAGO (0.7 nM) with DSLET (5 nM) binding were examined in thalamic membranes whereas [^{3}H]DSLET (1 nM) binding was determined in frontal cortical membranes. Results are expressed as percentage of control values based upon the means of triplicate samples. The study has been replicated three times.

the above issues regarding its selectivity must be considered in the design and interpretation of all studies.

Biochemically, the three sites differed. The most dramatic differences among the binding sites, however, was their sensitivity to magnesium. Magnesium had moderate effects on μ_2 and δ binding, raising binding up to 50%. μ_1 Binding was elevated almost 3-fold. Additional studies suggest that the increased μ_1 binding may represent interactions with G proteins, based upon its sensitivity towards GTP and its analogs.[1] Studies with trypsin and *N*-ethylmaleimide were not as clear. Although all three were inhibited by trypsin, the two μ sites were much more sensitive. Similarly, *N*-ethylmaleimide lowered μ binding more effectively than δ binding. Of the three, μ_1 binding was most affected.

In conclusion, the present binding experiments, along with pharmacological studies (10, 11, 14, 21, 22, 37), support the concept of two classes of μ receptors, μ_1 and μ_2. In the presence of DPDPE (10 nM), [^{3}H]DADL labeled μ_1 sites almost exclusively, leading us to use these conditions for a μ_1-selective binding assay. [^{3}H]DSLET gave binding results quite similar to those observed with [^{3}H]DADL and either radioligand could be used for the assay. In the past, detailed studies of μ_1 binding have been hindered by the low density of sites within the brain and the lack of a useful, selective radioligand. The availability of a simple μ_1 assay should facilitate additional studies.

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