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Opiate Receptor Binding: Effects of Enzymatic Treatments

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SUMMARY

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A variety of enzymatic treatments have been examined with regard to their effect on stereospecific [^{3}H]naloxone binding to rat brain homogenates. Opiate receptor binding is sensitive to very low concentrations of trypsin (EC 3.4.4.4) and chymotrypsin (EC 3.4.4.5). Trypsin appears to decrease the number of opiate receptor binding sites, while chymotrypsin primarily lowers their affinity for opiates. Receptor binding is drastically reduced by very low concentrations of phospholipase A (EC 3.1.1.4), is decreased by higher concentrations of phospholipase C (EC 3.1.4.3), and appears relatively insensitive to phospholipase D (EC 3.1.4.4) and neuraminidase (EC 3.2.1.18). Small amounts of RNase (EC 2.7.7.16) and DNase (EC 3.1.4.5) are without effect. Trypsin and chymotrypsin decrease receptor binding in a biphasic fashion, suggesting the presence of more than one population of sites sensitive to proteolysis. By contrast, the phospholipases degrade binding in a monophasic fashion. Thus the opiate receptor appears to be a membrane-bound complex whose stereospecific binding is dependent upon the integrity of both proteins and phospholipids.

INTRODUCTION

Recent studies have directly demonstrated specific opiate binding sites in nervous tissue (1), and these findings have been subsequently confirmed (2). Binding is stereospecific, an important criterion described by Goldstein *et al.* (3). Stereospecific [^{3}H]-naloxone binding to brain tissue is displaced by a variety of opiates and their antagonists at concentrations which parallel their pharmacological potency. [^{3}H]Naloxone-receptor complex formation fits models for a bimolecular reaction, and its dissociation follows first-order kinetics. From studies of the saturation of [^{3}H]naloxone binding, one can estimate 30 pmoles of receptor sites per gram of rat brain (4). Of 45 monkey and human brain regions studied, binding is greatest in areas in which morphine implantation most effectively elicits analgesia, and binding is unaffected by specific lesions of cholinergic, noradrenergic, and serotonergic pathways (5). While the identity of these binding sites with the pharmacologically relevant recognition site for opiates is supported by these data it is, conceivable that other opiate binding sites may exist with particular biological functions (3).

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We now report the influence of a variety of enzymes on opiate receptor binding in rat brain tissue.

MATERIALS AND METHODS

Trypsin (195 units/mg), trypsin treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK) (200 units/mg), phospholipase C (from *Clostridium welchii*, 1.5 units/mg), DNase I (2300 units/mg), chymotrypsin (60 units/mg), neuraminidase (1.13 units/mg), RNase (2700 units/mg), trypsinogen, and chymotrypsinogen were acquired from Worthington Biochemical Corporation. Phospholipase A (from *Vipera russelli*, 5.2 units/mg of protein; and bee venom, 1230 units/mg), phospholipase D (from cabbage, 22 units/mg), and Trizma buffer were purchased from Sigma Chemical Company, while phospholipase A (from *Crotalus terrificus terrificus*, 230 units/mg) was purchased from Calbiochem. "NCS" tissue solubilizer was obtained from Amersham/Searle. Naloxone was a generous gift of Endo Laboratories, and levorphanol and dextrorphan were donated by Roche Laboratories.

(−)-Naloxone was tritiated by New England Nuclear Corporation, using a tritium exchange method, and purified by thin-layer chromatography, as previously described (1). The specific activity was 5 Ci/mmole. In our system, 10^5 cpm of [^{3}H]naloxone represent 20 pmoles, assuming a counting efficiency of 44%.

Receptor binding assay. Male Sprague-Dawley rats (ASG Sprague-Dawley, Madison, Wis.) (180–220 g) were decapitated and their brains were rapidly removed. The cerebellum, which contains negligible binding (1), was excised, and the remainder of the brain was immediately placed in the appropriate volume of iced 50 mM Tris-HCl buffer (pH 7.7 at 25°; designated as standard buffer) and homogenized with a Brinkmann Polytron for 60 sec at setting 3. Unless otherwise stated, all subsequent procedures were performed at 4°. A typical filtration assay consisted of incubating 2 ml of tissue homogenate in triplicate with 5 mM EDTA and either levorphanol or dextrorphan at 100 nM for 5 min at 25°, adding [^{3}H]naloxone, and continuing the incubation for another 20 min. The incubation was terminated by filtration under vacuum over Whatman glass fiber filters (GF-B). The filters were then washed with two 5-ml volumes of cold standard buffer and placed in 15-ml aliquots of Triton X-100 scintillation fluor [250 ml of Triton X-100, 7.3 g of 2,5-diphenyloxazole (PPO), and 167 mg of *p*-bis[2-(5-phenyloxazolyl)]benzene (POPOP) in 1 liter of toluene]. The samples were shaken for 30 min and counted the next day on a Packard scintillation counter (model 3385) with an efficiency of 44%. Stereospecific binding is obtained by subtracting the binding in the presence of levorphanol from the binding in the presence of dextrorphan. Unless otherwise stated, all reported values of [^{3}H]-naloxone binding are stereospecific and are the means of triplicate determinations, which varied less than 10%.

Centrifugation experiments employed 2-ml aliquots of 1:10 (w/v) brain homogenates in standard Tris buffer. Preliminary incubation with 5 mM EDTA and either levorphanol or dextrorphan at 100 nM for 5 min at 25° was followed by a 15-min incubation after the addition of [^{3}H]naloxone. The samples were centrifuged at 49,000 × *g* for 10 min at 4° in a Sorvall ultracentrifuge. The pellets were dissolved in 1 ml of "NCS" tissue solubilizer and counted in 15 ml of toluene scintillation fluor (4 g of Omnifluor per liter of toluene).

The various conditions for enzymatic treatments are described under RESULTS. Enzymes were not further purified. Trypsin digestions performed either before or during the assay procedure, with or without EDTA, all give identical results. Sialic acid was measured by the thiobarbiturate assay of Warren (6).

To examine whether naloxone was altered during the binding procedure, 40 nM [^{3}H]-naloxone was bound to 10 ml of a 1:10 (w/v) homogenate of brain for 30 min at 25°, and the mixture was centrifuged at 49,000 × *g* for 40 min. The bound [^{3}H]-naloxone was recovered by immersing the pellet in a boiling water bath for 30 min. The mixture was again centrifuged at 49,000 × *g* for 40 min, and the supernatant fluid was used for binding. Heating authentic [^{3}H]-naloxone in standard buffer alongside the

bound [^{3}H]naloxone did not affect the binding of the former.

RESULTS

Factors influencing [^{3}H]naloxone binding assay. Filtration has been utilized for the standard assay for [^{3}H]naloxone binding in this laboratory. To explore the validity of this assay, we compared filtration with centrifugation as a means of recovering the [^{3}H]naloxone-receptor complex (Table 1). There is good agreement between the stereospecific [^{3}H]naloxone-receptor binding recovered by the two procedures, but the ratio of total binding to the "blank" value, obtained by measuring the binding of [^{3}H]naloxone in the presence of 100 nM levorphanol, is 2.4 times greater with filtration than with centrifugation. Because filtration results in lower blank values and can be accomplished more rapidly, we have chosen filtration as our standard assay. However, centrifugation appears to be a valid means of measuring opiate receptor binding.

TABLE 1

Comparison of centrifugation and filtration assays for specific [^{3}H]naloxone binding

Centrifugation assay: Rat brains, minus cerebella, were homogenized in 10 volumes (w/v) of standard Tris buffer. Two-milliliter aliquots were first incubated at 25° for 5 min with either levorphanol or dextrorphan at 100 nM, followed by a 15-min incubation at the same temperature after the addition of 20 nM [^{3}H]naloxone. The tissue was recovered by centrifugation at 49,000 × *g* for 10 min at 4°. The pellet was dissolved in "NCS" and counted with toluene phosphor. Values represent results for 10 mg of tissue, wet weight. Stereospecific binding is defined as the binding in the presence of dextrorphan minus the binding in the presence of levorphanol. All values are the mean of triplicate determinations.

Filtration assay: Rat brains, minus the cerebella, were homogenized in 100 volumes (w/v) of standard Tris buffer. Two-milliliter samples were then incubated for 5 min with either levorphanol or dextrorphan at 100 nM, followed by another incubation at 25° for 15 min after the addition of 20 nM [^{3}H]naloxone. The samples were filtered and counted as described in MATERIALS AND METHODS. Values represent results for 10 mg of tissue, wet weight. Stereospecific binding is defined as the binding in the presence of dextrorphan minus the binding in the presence of levorphanol. All values are the means of triplicate determinations. The experiment was repeated three times. Variance was less than 6% in all triplicate determinations.

Assay	[^{3}H]Naloxone binding		
	Levorphanol	Dextrorphan	Stereospecific
	cpm	*cpm*	*cpm*
Centrifugation	2367	4212	1845
Filtration	553	2437	1884

In earlier studies of opiate-receptor binding, incubations were carried out at 35° (1, 4). Because of the possibility of degradation of the receptor complex during the prolonged incubations needed for enzymatic treatments, we chose to conduct assays at 25°. As had been observed previously at 35°, binding of [^{3}H]naloxone to brain tissue occurred rapidly at 25° and equilibrium was reached by 10 min. There did not appear to be significant degradation of the [^{3}H]naloxone-receptor complex even when incubations were extended to 60 min (Fig. 1). Under these assay conditions, stereospecific [^{3}H]naloxone binding was linear with tissue concentration to at least 20 mg of tissue (wet weight) per 2 ml (Fig. 2).

Because of the prolonged incubations required for enzymatic treatments, we examined the thermal stability of the receptor by incubating brain tissue for periods up to 60 min at either 37° or 25° before conducting the standard assay at 25° (Fig. 3). Although stereospecific binding declined at both temperatures, the decrease at 37° was much more pronounced. A 60-min prior incubation at 37° followed by 40 min at 25° decreased binding by almost 50%, while maintaining the homogenate at 25° for 100 min reduced binding only 19%.

Previous studies have shown that the [^{3}H]naloxone recovered after binding migrates identically with authentic naloxone on thin-layer chromatography (1). To examine for subtle changes, [^{3}H]naloxone was bound to tissue, dissociated, and bound again. The stereospecific binding of the recovered radioactivity was 0.139 pmole/sample, while a control of untreated [^{3}H]naloxone yielded 0.131 pmole/sample (both are means of triplicate determinations).

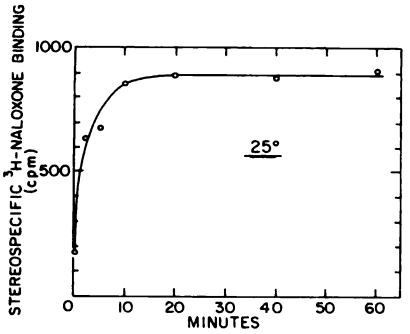

FIG. 1. *Time course of [^{3}H]naloxone binding at 25°*

Rat brains, minus cerebella, were homogenized in 120 volumes (w/v) of standard Tris buffer. Two-milliliter aliquots were first incubated at 25° for 10 min with 5 mM EDTA and either levorphanol or dextrorphan at 100 nM, and then incubated for the indicated time at 25° in the presence of 6 nM [^{3}H]naloxone. The samples were filtered and counted as described in MATERIALS AND METHODS. All values are the means of triplicate determinations, with less than 8% variability. The experiment was repeated three times.

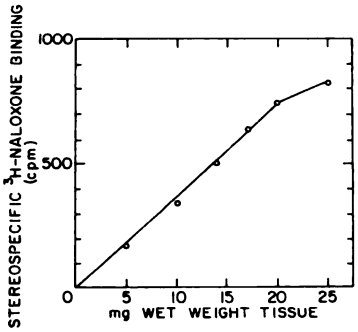

FIG. 2. *Linearity of [^{3}H]naloxone binding with respect to tissue concentration*

Rat brains, minus cerebella, were homogenized in 50 volumes (w/v) of standard Tris buffer. Appropriate amounts of homogenate were diluted to a final volume of 2 ml. The samples were then incubated for 10 min at 25° with 5 mM EDTA and either levorphanol or dextrorphan at 100 nM, and incubated for another 30 min at 25° with 10 nM [^{3}H]naloxone. The samples were filtered and counted as described in MATERIALS AND METHODS. All values are the means of triplicate determinations, which varied less than 10%, and are expressed as milligrams of tissue, wet weight, per 2-ml aliquot. The experiment was repeated four times.

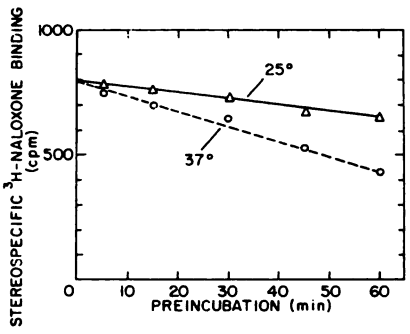

FIG. 3. *Effect of incubation at 25° vs. 37° on specific [^{3}H]naloxone binding*

Rat brains, minus cerebella, were homogenized in 120 volumes (w/v) of standard Tris buffer. The homogenate was incubated at the designated temperature for the indicated time in the absence of all drugs. Unincubated samples were kept in an ice-water bath. After the preliminary incubation without drugs, 2-ml aliquots of homogenate were incubated at 25° for 10 min in the presence of 5 mM EDTA and either levorphanol or dextrorphan at 100 nM, and the incubation was continued for 30 min after the addition of 6 nM [^{3}H]naloxone. Samples were filtered and counted as described in MATERIALS AND METHODS. All values are the means of triplicate determinations, which varied less than 6%. The experiment was repeated twice.

Previously we found that 5 mM calcium reduced specific [^{3}H]naloxone binding about 50% (4). EDTA maximally enhanced specific [^{3}H]naloxone binding 55% at 5 mM concentration and caused a 25% increase at 2 mM.[3] Accordingly, 5 mM EDTA was included routinely in most subsequent assays. A Scatchard plot under these conditions defines a straight line, strongly suggesting a single species of receptor with a dissociation constant (K_D) of 67 nM and a concentration of 9 pmoles/g of tissue, wet weight (Fig. 4), similar to a previously reported value of 30 pmoles/g, wet weight (4).

Influence of trypsin and phospholipase C upon specific [^{3}H]naloxone binding in various subcellular fractions of rat brain. Digestion with phospholipase C was performed on the crude nuclear (P_1), crude mitochondrial (P_2), and crude microsomal (P_3) fractions for 15 min at 37°, followed by the standard assay at 25° in the continued presence of the

[3] G. W. Pasternak and S. H. Snyder, manuscript in preparation.

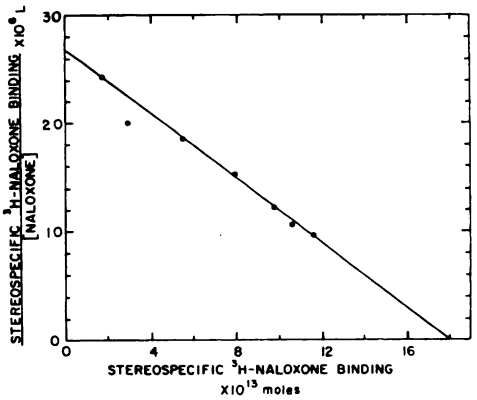

FIG. 4. *Scatchard plot of [³H]naloxone binding to rat brain homogenates*

Rat brains, minus cerebella, were homogenized in 100 volumes (w/v) of standard Tris buffer. Two-milliliter aliquots were first incubated at 25° for 5 min with 5 mM EDTA and levorphanol or dextrorphan at 100 nM, and then incubated at 25° for 30 min with the appropriate amount of [³H]naloxone. [³H]Naloxone binding is expressed in moles, and free naloxone in moles per liter. The samples were filtered and counted as described in MATERIALS AND METHODS. All values are the means of triplicate determinations, with under 8% variability. The experiment was repeated four times.

enzyme. No prior incubation with trypsin was performed, because proteolysis could be achieved at 25°. Thus trypsin was included in the standard [³H]naloxone binding assay at 25° (Table 2). In all three subcellular fractions trypsin reduced binding about 50% while phospholipase C decreased binding about 65%. Since a significant amount of binding occurs in all three subcellular fractions and they are affected by these enzymes to a similar extent, subsequent studies of the influence of enzymes upon opiate receptor binding were conducted with homogenates of whole rat brain minus cerebella.

Influence of various amounts of proteolytic enzymes on specific [³H]naloxone binding. The influence of trypsin on opiate receptor binding was examined in several ways. In one approach, brain homogenate was incubated without EDTA for 30 min at 25° with concentrations of trypsin ranging from 0.5 to 30 μg/ml. This treatment was followed by a 10-min incubation at 25° with either levorphanol or dextrorphan at 100 nM, after

TABLE 2

Effects of trypsin and phospholipase C on stereospecific [³H]naloxone binding of brain subcellular fractions

Rat brains, minus cerebella, were homogenized in 10 volumes (w/v) of 0.32 M sucrose with a Teflon pestle. The homogenate was centrifuged at 1000 × *g* for 10 min, and the pellet (P_1) was resuspended in 10 times the original volume of standard Tris buffer. The supernatant fraction was then centrifuged at 20,000 × *g* for 20 min, and the pellet (P_2) was resuspended in 10 times the original volume of standard Tris buffer. The supernatant fraction (S_2) was diluted 10 times with standard Tris buffer. Since the assay measures only particulate matter and not soluble receptor, S_2 is the equivalent of the crude microsomal fraction, P_3. Aliquots of each fraction were treated with 2 μg/ml of trypsin during the standard incubation, or 150 μg/ml of phospholipase C for 15 min at 37° before the standard incubation, or no enzyme (controls). The standard incubation consisted of 5 min at 25° with 5 mM EDTA and either levorphanol or dextrorphan at 100 nM, followed by another 20 min after the addition of 10 nM [³H]naloxone. The samples were filtered and counted as described in MATERIALS AND METHODS. All values are the means of triplicate determinations, which varied less than 9%. Values represent the total receptor binding in each subcellular fraction. The experiment was performed twice.

Fraction	Stereospecific [³H]naloxone binding		
	Control	Trypsin treatment (% control)	Phospholipase C treatment (% control)
	cpm × 10^{-4}	*cpm* × 10^{-4}	*cpm* × 10^{-4}
P_1	2.19	1.18(58%)	0.69(38%)
P_2	3.59	1.68(47%)	1.23(34%)
S_2	1.34	0.64(48%)	0.50(37%)

which [³H]naloxone was added and the incubation was continued for another 30 min (Fig. 5). As little as 0.5 μg/ml of trypsin reduced binding by 40%. As the trypsin concentration was increased it appeared to destroy receptor binding in more than one phase. Using a semilogarithmic plot, one sees a very sensitive phase up to 1 μg/ml and a less sensitive phase between 1 and 30 μg/ml. Almost all receptor binding was destroyed at 15 μg/ml.

A similar relationship between trypsin concentration and receptor binding was ob-

tained in other experiments, in which EDTA (5 mM) was included during the incubation with trypsin as well as during the standard assay. Moreover, opiate receptor binding showed a similar biphasic sensitivity to trypsin when the enzyme was included only during the standard assay for receptor binding. Experiments involving varying durations of incubation indicated that the effects of trypsin (10 or 20 μg/ml) on the opiate receptor were virtually complete after 5 min. Trypsin treated with TPCK to destroy chymotryptic activity gives the same results as untreated trypsin. Specific [^{3}H]naloxone binding was also sensitive to treatment with α-chymotrypsin (Fig. 6). As with trypsin, the degradation was biphasic, with a very sensitive phase up to 0.5 μg/ml and a less sensitive phase between 0.5 and 250 μg/ml. About 50% loss of binding occurred at 0.5

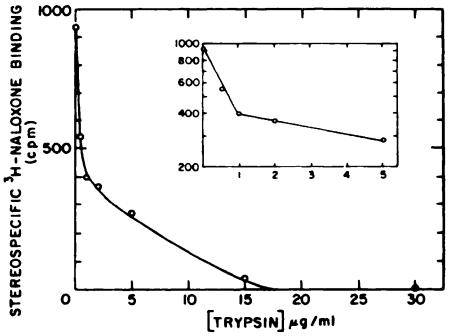

FIG. 5. *Influence of varying amounts of trypsin on specific [^{3}H]naloxone binding to rat brain homogenates*

Rat brains, minus cerebella, were homogenized in 100 volumes (w/v) of standard Tris buffer. Two-milliliter aliquots were incubated at 25° with the indicated amount of trypsin for 30 min. Another incubation with 5 mM EDTA and either levorphanol or dextrorphan at 100 nM at 25° for 10 min was followed by a third at the same temperature for 40 min in the presence of 6 nM [^{3}H]naloxone. The samples were filtered and counted as described in MATERIALS AND METHODS. Subsequent experiments showed that in this system, performing the trypsin digestion in the presence of drugs and EDTA at the same time as the binding assay yields the same results. All values are the means of triplicate determinations, which varied less than 9%. The experiment was repeated three times.

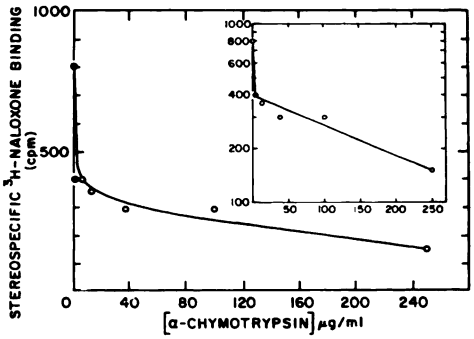

FIG. 6. *Influence of varying amounts of chymotrypsin on [^{3}H]naloxone binding by rat brain homogenates*

Rat brains, minus cerebella, were homogenized in 100 volumes (w/v) of standard Tris buffer. Two-milliliter aliquots were treated with the designated amount of enzyme at 25° for 30 min. The samples were then incubated with 5 mM EDTA and either levorphanol or dextrorphan at 100 nM at 25° for 5 min, and then incubated for 30 min at the same temperature in the presence of 6 nM [^{3}H]naloxone. The samples were filtered and counted as described in MATERIALS AND METHODS. All values are the means of triplicate determinations, which varied less than 10%. The experiment was repeated three times.

μg/ml. Unlike results obtained with trypsin, we did not observe total loss of binding even at 250 μg/ml of α-chymotrypsin. Neither trypsin nor chymotrypsin altered nonspecific binding at any concentration examined.

Influence of phospholipases on opiate receptor binding. Specific [^{3}H]naloxone binding was extremely sensitive to degradation by commercial phospholipase A from *V. russelli* (Fig. 7). Receptor binding was completely destroyed by 0.25 μg/ml of the enzyme, and half-maximal destruction occurred at less than 0.05 μg/ml. Unlike results with trypsin and chymotrypsin, degradation of the receptor by phospholipase A appeared to be monophasic when plotted semilogarithmically. Phospholipases A from three different sources were tested with regard to calcium dependence and stability to heating (Table 3). Since crude homogenates were used, EDTA was added to the control enzyme degradations to chelate endogenous calcium. All three preparations of phospho-

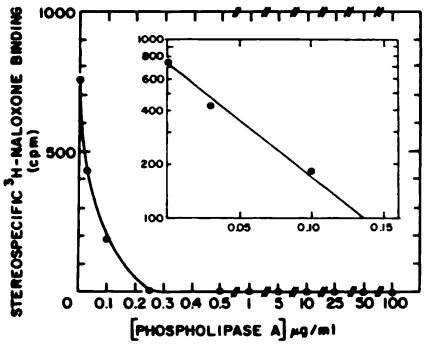

FIG. 7. *Effect of varying amounts of phospholipase A on [^{3}H]naloxone binding to rat brain homogenates*

Rat brains, minus cerebella, were homogenized in 100 volumes (w/v) of standard Tris buffer and digested with the stated amount of untreated enzyme in the presence of 5 mM $CaCl_2$ at 25° for 30 min. Then 2-ml aliquots were incubated for 5 min at 25° with 5 mM EDTA and either levorphanol or dextrorphan at 100 nM, followed by a 30-min incubation at 25° in the presence of 10 nM [^{3}H]-naloxone. The samples were filtered and counted as described in MATERIALS AND METHODS. All values are the means of triplicate determinations, which varied less than 10%. The experiment was repeated three times.

lipase A elicited a marked reduction of binding. The action of all three enzymes was calcium-dependent and resistant to a 7-min treatment in a boiling water bath. Lysolecithin decreased binding when present during the assay, but 100 μg/ml (0.2 mM) concentrations were necessary to decrease binding 50%. Oleic acid (0.05 mM) had no effect on stereospecific opiate binding.

Receptor binding was considerably less sensitive to treatment with phospholipase C (Fig. 8). As with phospholipase A, the reduction of binding was monophasic with increasing enzyme concentration. Specific [^{3}H]-naloxone binding was reduced 40% by 150 μg/ml of phospholipase C. Phospholipase D failed to reduce specific receptor binding significantly at concentrations ranging from 1 to 400 μg/ml. None of the phospholipases altered nonspecific binding in any concentration studied.

Enzymatic treatments which do not alter opiate receptor binding. Concentrations of neuraminidase as high as 50 μg/ml failed to alter specific [^{3}H]naloxone binding despite the fact that over 98% of the reported membrane-bound sialic acid was released into the soluble supernatant fluid (Fig. 9) (7). RNase and DNase at 100 μg/ml failed to alter [^{3}H]naloxone binding. The slight decreases observed with trypsinogen and chymotrypsinogen (Table 4) might have been due to the presence of small amounts of trypsin and chymotrypsin in these preparations.

TABLE 3

Calcium dependence and effects of heating of phospholipase A upon stereospecific [^{3}H]naloxone binding

Each phospholipase A solution was divided into two portions, one of which was placed in a boiling water bath for 7 min while the other was kept on ice. Both portions were then tested with and without 5 mM calcium chloride. To chelate endogenous calcium, 5 mM EDTA was added to the calcium-free samples. Rat brains, minus cerebella, were homogenized in 120 volumes (w/v) of standard Tris buffer. Aliquots were taken and incubated at 25° for 30 min with the appropriate additions. The digested samples were then centrifuged at 49,000 × *g* for 20 min in a Sorvall centrifuge at 4°. The supernatant fraction was discarded, and the pellets were resuspended in the original volume of standard Tris buffer. Two-milliliter samples were then incubated at 25° for 5 min with 5 mM EDTA and either levorphanol or dextrorphan at 100 nM. [^{3}H]Naloxone at 10 nM was added, and the incubation was continued for another 20 min. Samples were filtered and counted as described in MATERIALS AND METHODS. The values are means of triplicate determinations, which varied less than 8%. All experiments were repeated at least twice.

Additions	Stereospecific [^{3}H]naloxone binding		
	V. russelli (1 μg/ml)	Bee venom (1 μg/ml)	*C. terrificus terrificus* (2.5 μg/ml)
	cpm	*cpm*	*cpm*
None	1118	1013	1205
Enzyme + 5 mM $CaCl_2$	0	0	383
Enzyme + 5 mM EDTA	1195	826	1250
Boiled enzyme + 5 mM $CaCl_2$	0	0	842
Boiled enzyme + 5 mM EDTA	1177	853	1197

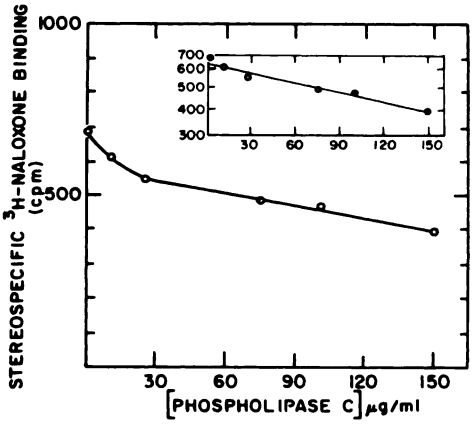

FIG. 8. *Effect of varying amounts of phospholipase C on specific [^{3}H]naloxone binding to rat brain homogenates*

Rat brains, minus cerebella, were homogenized in 100 volumes (w/v) of standard Tris buffer and incubated with the appropriate amount of enzyme for 15 min at 37°. Two-milliliter aliquots were then incubated with 5 mM EDTA and either levorphanol or dextrorphanol at 100 nM for 5 min at 25°, and the incubation was continued at 25° for 30 min after the addition of 6 nM [^{3}H]-naloxone. The samples were filtered and counted as described in MATERIALS AND METHODS. All values are the means of triplicate determinations, which varied less than 7%. The experiment was repeated three times.

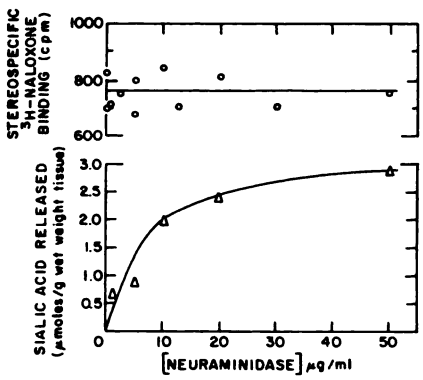

FIG. 9. *Effect of neuraminidase on [^{3}H]naloxone binding and release of sialic acid in rat brain homogenates*

Rat brains, minus cerebella, were homogenized in 100 volumes (w/v) of standard Tris buffer and treated with the stated amount of enzyme at 37° for 15 min. Samples were analyzed for [^{3}H]naloxone binding with a 5-min preliminary incubation at 25° with 5 mM EDTA and either levorphanol or dextrorphan at 100 nM and a 20-min incubation at 25° after the addition of 6 nM [^{3}H]naloxone. The samples were filtered and counted as described in MATERIALS AND METHODS. Portions of digested homogenates were centrifuged at 49,500 × *g* for 45 min, and an aliquot of the supernatant fluid was analyzed for unbound sialic acid, using the thiobarbiturate assay of Warren (6). The levels of sialic acid in undigested homogenates were subtracted from values for digested homogenates, to obtain the amount of sialic acid released. The values are the means of triplicate determinations, which varied less than 8%. The experiment was repeated twice.

Effects of trypsin, chymotrypsin, phospholipase C, and phospholipase A upon opiate receptor binding at various [^{3}H]naloxone concentrations. To assess whether enzymatic treatment would alter the total number of receptors or their affinity, we measured the ability of brain homogenates to bind various concentrations of [^{3}H]naloxone after treatment with trypsin, chymotrypsin, phospholipase C (Fig. 10), or phospholipase A (Fig. 11). Saturation curves were then fitted to a Scatchard plot by the method of least squares, and their zero-order correlation coefficient was determined (Table 5). All correlation coefficients were at least 0.85. Digestion with trypsin (0.25 μg/ml and 2 μg/ml), phospholipase A (0.03 μg/ml), and phospholipase C (25 μg/ml) produced little change in the K_D, but significant decrements in the total number of receptor sites.

By contrast, receptor binding with increasing concentrations of [^{3}H]naloxone in chymotrypsin-treated brain homogenates did not reach a plateau as rapidly as did untreated homogenates. The K_D of the chymotrypsin-treated sites was 2-fold higher than the control, while the decrease in the total number of receptor sites was very small.

DISCUSSION

At very low concentrations, trypsin and chymotrypsin markedly reduced opiate receptor binding, indicating that protein is an important component of the opiate-receptor complex. The opiate receptor appears to be more sensitive to trypsin and chymotrypsin

TABLE 4

Effects of DNase, RNase, trypsinogen, and chymotrypsinogen on stereospecific [³H]naloxone binding

DNase: Rat brains, minus cerebella, were homogenized in 100 volumes (w/v) of standard Tris buffer. The incubation with 50 μg of DNase per milliliter had 10 mM $MgCl_2$, and the incubation with 100 μg of DNase per milliliter had 20 mM $MgCl_2$. The homogenate was incubated at 37° for 15 min with the appropriate amount of enzyme.

RNase: Rat brains, minus cerebella, were homogenized in 100 volumes of standard Tris buffer and incubated at 37° for 15 min with the appropriate amount of enzyme.

Trypsinogen and chymotrypsinogen: Rat brains, minus cerebella, were homogenized in 100 volumes (w/v) of standard Tris buffer, and the appropriate amount of enzyme precursor was then added. Two-milliliter aliquots of all enzymatically treated tissues were first incubated at 25° for 5 min with the appropriate amount of enzyme precursor, 5 mM EDTA, and either levorphanol or dextrorphan at 100 nM, followed by a 30-min incubation after the addition of nM [³H]naloxone The samples were filtered and counted as described in MATERIALS AND METHODS. The values are means of triplicate determinations, which varied less than 7% for all values. The experiment was repeated twice.

Addition	Concentration	Stereospecific [³H]naloxone binding
	μg/ml	*cpm*
DNase	0	793
	50	815
	0	1030
	100	1073
RNase	0	707
	50	664
	0	945
	100	1060
Trypsinogen	0	823
	10	875
	25	778
Chymotrypsinogen	0	1140
	10	1050

than the insulin receptor (8). In contrast to the virtually complete degradation of the opiate receptor by trypsin, chymotrypsin failed to abolish opiate receptor binding even in high concentrations. These differences may be related to the action of the enzymes. Chymotrypsin seems to alter the affinity of the receptor with little effect upon the total number of receptors, while trypsin destroys the receptor without altering its affinity for [³H]naloxone. The biphasic decrease in binding shown by both trypsin and chymotrypsin suggests the presence of more than one population of receptor sites with varying degrees of sensitivity to proteolysis, possibly on account of differences in accessibility of the enzyme to the receptor. However, with crude systems such as ours, the interpretation of such biphasic effects is difficult.

Receptor binding is also very sensitive to phospholipase A. Phospholipase A activity is stable to boiling at acid pH, while most contaminating enzymes are not (9). In addition, phospholipase A requires calcium for activity. Degradation of binding by all three phospholipase A preparations tested was relatively resistant to boiling and showed complete calcium dependence for both boiled and nonboiled enzymes. Lysolecithin, a product of the enzymatic digestion, does inhibit binding, but at high concentrations (ID_{50} = 0.2 mM), while oleic acid, the other major product, has no effect. This suggests that the effects of phospholipase A are attributable to its degradation of a lipid component of the receptor. The apparent role of phospholipids in opiate receptor binding is supported by its extreme sensitivity to detergents such as Triton X-100, sodium dodecyl sulfate, and deoxycholate, which fully abolish binding at 0.01% concentrations,[3] similar to detergent effects on another apparent opiate-binding protein (10). These results suggest that the effects are attributable to the degradation of a lipid component of the receptor. It is extremely difficult to differentiate between direct effects on the receptor complex and indirect, general membrane effects. However, the extremely low concentrations of enzymes used and the

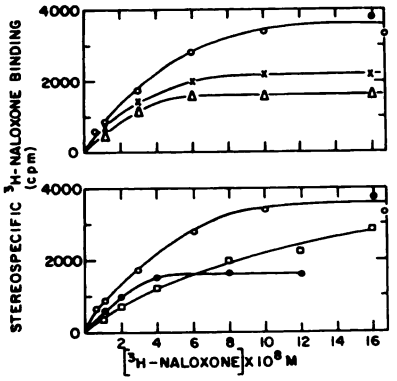

FIG. 10. *Naloxone saturation curves for rat brain homogenates treated with trypsin, chymotrypsin, and phospholipase C*

All digestions and assays were performed with rat brains, minus cerebella, homogenized in 100 volumes (w/v) of standard buffer.

Untreated samples (○): One milliliter of homogenate was first incubated for 10 min with 5 mM EDTA and either levorphanol or dextrorphan at 100 nM, and then incubated at 25° for 30 min after the addition of the specified amount of [^{3}H]naloxone.

Trypsin digest (×, 2 μg/ml; △, 0.25 μg/ml): One milliliter of homogenate was first incubated with 5 mM EDTA and either levorphanol or dextrorphan at 100 nM at 25° for 10 min, and then incubated for 30 min at the same temperature after the addition of the appropriate amounts of trypsin and [^{3}H]naloxone.

Chymotrypsin digest (□): The homogenate was digested with 5 μg/ml of chymotrypsin for 30 min at 25°. One-milliliter aliquots were then incubated in the presence of 5 mM EDTA and either levorphanol or dextrorphan at 100 nM at 25° for 5 min, followed by a 30-min incubation at 25° after the addition of the appropriate amount of [^{3}H]naloxone. Additional incubations, which were conducted identically except for omission of the enzymes, gave the same results as control incubations (○).

Phospholipase C (●): The homogenate was digested with 25 μg/ml of phospholipase C at 37° for 15 min. One-milliliter aliquots were first incubated in the presence of 5 mM EDTA and either levorphanol or dextrorphan at 100 nM at 25° for 5 min, and incubated for 30 min at the same temperature after the addition of the appropriate amount of [^{3}H]naloxone. All samples were filtered and counted as described in MATERIALS AND METHODS. All values are the means of triplicate determinations, which varied less than 7%.

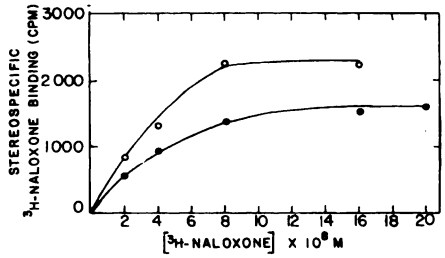

FIG. 11. *Naloxone saturation curves for rat brain homogenates treated with phospholipase A*

Rat brains, minus cerebella, were homogenized in 60 volumes (w/v) of standard Tris buffer with 5 mM $CaCl_2$. Half of the homogenate (●) was digested at 25° for 30 min with 0.03 μg/ml of phospholipase A from *V. russelli* and then centrifuged at 49,000 × *g* for 20 min. The other half (○) was incubated at 25° for 30 min with no enzyme and centrifuged at 49,000 × *g* for 20 min. The pellets were resuspended in twice the original volume of standard Tris buffer. Two-milliliter aliquots of the untreated and the phospholipase A-treated homogenates were incubated at 25° for 5 min with 5 mM EDTA and either levorphanol or dextrorphan at 100 nM, and the incubation was continued for 20 min after the addition of the appropriate amount of [^{3}H]naloxone. All samples were filtered and counted as described in MATERIALS AND METHODS. All values are the means of triplicate determinations, which varied less than 8%. The experiment was repeated three times.

totally reversible inhibition of binding by very low concentrations of detergents and organic solvents[3] favor a specific effect.

Phospholipase C from *C. welchii* was considerably less active than phospholipase A. Since this enzyme has not been extensively purified, it is possible that contaminating enzymes may play a role in its actions. Phospholipase D had no effect on receptor binding. These results suggest that the polar moieties of phospholipids, which are affected selectively by phospholipases C and D, may not be required for binding. The failure of neuraminidase to alter binding implies that sialic acid residues are not essential for [^{3}H]-naloxone binding. DNase and RNase were

All experiments were performed at least twice. Additional incubations, which were conducted identically except for omission of enzymes, gave the same results as control incubations (○).

TABLE 5

Effects of enzyme on apparent number of binding sites in rat brain homogenates and their affinity for opiates

The [^{3}H]naloxone saturation curves presented in Figs. 10 and 11 were fitted to a Scatchard plot, using a least-squares linear regression analysis (program code 15.07) in an Olivetti Underwood Programma 101. The K_D was calculated from the slope, and the maximal [^{3}H]naloxone binding from the slope and the y intercept. Data are presented as the maximal [^{3}H]naloxone binding in 10 mg of tissue, wet weight. A zero-order correlation coefficient was also determined.

Treatment	Correlation coefficient	K_D	Maximal [^{3}H]naloxone binding
		$\times 10^{9} M$	$\times 10^{13}$ moles
None	0.96	4.6	9.7
Trypsin (0.25 μg/ml)	0.88	3.7	6.5
Trypsin (2.0 μg/ml)	0.87	4.7	4.8
Phospholipase C	0.88	3.3	4.8
Chymotrypsin	0.95	9.2	8.4
None	0.85	6.8	7.4
Phospholipase A	0.97	5.5	4.5

also without effect. In addition, purified nuclei exhibited no stereospecific binding, while the separated debris from crude nuclear pellets did show stereospecific opiate binding.[3]

Simon *et al.* (2) reported that high concentrations of trypsin reduced stereospecific [^{3}H]etorphine binding to rat brain homogenates 80–90%, while phospholipases A and C had negligible effects. Their failure to detect an effect with phospholipase A, in contrast to the profound reduction in binding we observed, may relate to their possible omission of calcium, which is required for phospholipase A activity.

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