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Stereospecific Binding of the Potent Narcotic Analgesic [^{3}H]Etorphine to Rat-Brain Homogenate

(opiate receptor/morphine/antagonist)

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ABSTRACT **Etorphine, the most potent narcotic analgesic known, was labeled with tritium by catalytic exchange. This drug exhibits stereospecific, saturable binding to rat-brain homogenate. At saturation, the stereospecific binding is 0.1–0.15 pmol/mg of protein. Specific binding is inhibited by high salt concentrations, sulfhydryl reagents, and proteolytic enzymes, but is unaffected by phospholipases A and C, sodium azide, sodium fluoride, and prostaglandins E_1 and E_2. Competition for binding of [^{3}H]etorphine correlates with agonist and antagonist potencies. The stable, stereospecific binding of an active narcotic analgesic supports the existence of opiate receptors.**

Specific receptors for opiates in the central nervous system have been postulated for many years, based primarily on the strict stereospecificity of narcotic analgesic action and the relatively minor structural alterations that result in formation of highly specific antagonists.

Earlier attempts in our laboratory (1) to detect opiate receptors by measuring nalorphine-releasable binding of morphine to brain homogenate were unsuccessful. Goldstein *et al.* (2) reported the stereospecific binding of labeled levorphanol to mouse-brain homogenate. This was defined as the difference between the binding observed in the presence of 100-fold excess of unlabeled levorphanol and of its inactive enantiomorph dextrorphan. However, the specific binding amounted to only 2% of the total binding. Attempts to repeat these experiments in our laboratory were unsuccessful.

Pert and Snyder (3), using a modification of the Goldstein procedure, reported the stereospecific binding of [^{3}H]naloxone, a potent narcotic antagonist, to rat-brain homogenate. The binding was observed exclusively in nervous tissue. The modification of the Goldstein procedure used by these authors and, independently, in our laboratory involves the use of very low concentrations of labeled ligand made possible by very high specific radioactivity, and washing of the homogenates after incubation with cold buffer to remove contaminating unbound and loosely bound radioactivity. The specific binding is of sufficient affinity to withstand the cold washes and constitutes a major portion of the total residual binding.

We first used this procedure to study the binding of [^{3}H]-dihydromorphine of very high specific activity (20 Ci/mmol), but have never been able to demonstrate specific binding of this drug. We have recently obtained etorphine, the most potent morphine-type analgesic known. The drug was labeled with tritium by catalytic exchange. The resulting [^{3}H]-etorphine exhibits stereospecific binding to rat-brain homogenate. The amount and most of the properties of the binding are similar to those reported for the antagonist naloxone by Pert and Snyder (3).

The present report provides further support for the existence of opiate receptors and furnishes evidence that stable binding to receptors is not an exclusive property of antagonists. We report several new properties of the receptors and some differences from the results reported for naloxone binding.

MATERIALS AND METHODS

Materials. Etorphine was purchased from American Cyanamide. Levorphanol and dextrorphan were generously donated by Hoffman–LaRoche, Inc., morphine and nalorphine by Merck & Co., naloxone by Endo Laboratories, and methadone by Eli Lily. Prostaglandins E_1 and E_2 were made available by Dr. J. Pike, Upjohn Co.

Iodoacetamide and *p*-hydroxymercuribenzoate were obtained from Sigma Chemical Co., Pronase and trypsin from Calbiochem., *N*-ethylmaleimide from General Biochemicals, and sodium fluoride from Fisher Scientific. Phospholipase A was prepared from *Crotalus adamanteus* venom (Batch no: 40582, Koch–Light Laboratories) by heat denaturation of contaminating enzymes as described by Blecher (4). The enzyme was highly active with labeled *Escherichia coli* phospholipids as substrate. Phospholipase C (lot no. P-7633, Sigma) was Type 1 from *Cl. welchii.*

Preparation of [^{3}H]Etorphine. Etorphine, free base, was obtained from a solution of etorphine hydrochloride by neutralization with 2 N NaOH to pH 7. The precipitated base was removed by sedimentation, washed with water, and dried. A sample was labeled with tritium by New England Nuclear as follows: Etorphine (12 mg) was dissolved in 1 ml of ethylacetate. To this was added 10 mg of 10% palladium on charcoal catalyst and 5 Ci of tritium gas.* The reaction mixture was stirred overnight at room temperature. Labile tritium was removed under reduced pressure with ethanol as solvent. After filtration, the product was taken up in 10 ml of ethanol. Specific activity was 3.3 Ci/mmol. The product gave a single radioactive peak identical to that of unlabeled etorphine when chromatographed on silica gel plates with the solvent ethanol–*p*-dioxane–benzene–ammonium hydroxide 40:5:50:5 (5).

Binding Assays. Male Sprague–Dawley rats were decapitated. The brain was removed quickly and homogenized at

* The double bond is not reduced under these conditions since high temperature and pressure are required for the hydrogenation of etorphine [Bentley, K. W., Hardy, D. G. & Meek, B. (1967) *J. Amer. Chem. Soc.* **89**, 3273–3280].

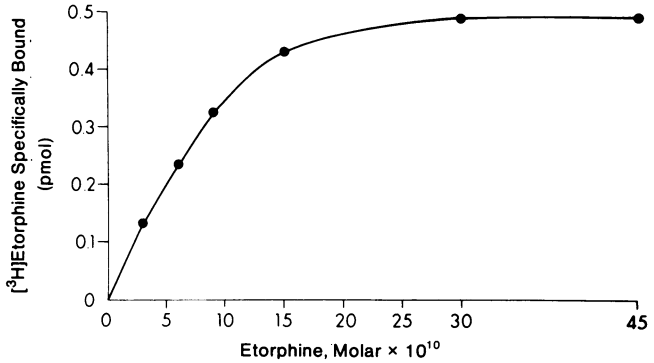

FIG. 1. Saturation curve for the specific binding of [^{3}H]-etorphine to rat-brain homogenate. The concentration of dextrorphan or levorphanol present during preincubation was 10^{-7} M.

4° in 6 volumes of 0.05 M Tris·HCl buffer (pH 7.4). Homogenates were diluted further with buffer to give suitable concentrations of protein. 2 ml of homogenate were first incubated for 5 min at 37° with a competing unlabeled drug, followed by a 15-min incubation with [^{3}H]etorphine. In earlier experiments a centrifugation procedure similar to that of Goldstein (2) was used, except that pellets were washed twice with cold 0.05 M Tris·HCl buffer. More recently, we adopted the more rapid filtration procedure used by Pert and Snyder (3). Samples were filtered through Whatman GF/B filters, 2.4 cm in diameter, and washed twice with 4-ml volumes of cold Tris·HCl buffer (determined by a washing curve that showed no further decrease of radioactivity on the filter after the first wash). The concentrations of drugs and amounts of homogenate protein are indicated in legends to the figure and tables. All samples were run in triplicate. Protein was determined by the method of Lowry *et al.* (6). Filters were dried under an infrared lamp and counted in 10 ml of toluene scintillation cocktail in a Packard Tricarb scintillation counter.

RESULTS

Stereospecific, Saturable Binding of [^{3}H]Etorphine to Rat-Brain Homogenate. Incubation of rat-brain homogenate with [^{3}H]etorphine of high specific activity at concentrations around 10^{-9} M results in significant binding (Table 1), much of which is stereospecific as defined by Goldstein *et al.* (2), i.e., it is prevented by levorphanol but not by its inactive enantiomorph, dextrorphan. The same reduction in binding is observed in the presence of the antagonist, naloxone. Specific binding is 40–60% of the total and represents 0.1–0.15 pmol/mg of protein for whole-brain homogenate. Binding is most rapid at 37°, is linear for 3 min, and complete in 10 min. Fig. 1 shows that the specific binding of etorphine is saturable at 2×10^{-9} M, with half saturation occurring at 6×10^{-10} M. The binding is proportional to the amount of protein in the homogenate in the range studied (1–4 mg of protein in 2 ml). Addition of nonradioactive etorphine (10^{-7} M) or naloxone (10^{-6} M) after binding of [^{3}H]etorphine is complete, causes rapid release of stereospecifically bound radioactivity. No stereospecific binding was observed with liver homogenate under these conditions.

TABLE 1. *Binding of [^{3}H]etorphine to rat-brain homogenate*

Unlabeled drug in preincubation	cpm ± SD	pmol bound
None	1434 ± 38	1.1
Dextrorphan (D)	1477 ± 29	1.1
Levorphanol (L)	895 ± 55	0.7
Naloxone (N)	807 ± 34	0.6
Specific binding		
D-L	582	0.45
D-N	670	0.5

2 ml of homogenate contained 3.6 mg of protein. [^{3}H]etorphine: 3×10^{-9} M, 3 Ci/mmol. The concentration of unlabeled drugs was 10^{-7} M. Results of a representative experiment are given as the mean ± SD of triplicate determinations.

Properties of Binding. Specific binding of [^{3}H]etorphine has a broad pH optimum between 6.5 and 8. Contrary to the observations of Pert and Snyder (3) with naloxone, Table 2 demonstrates that specific binding of [^{3}H]etorphine is quite sensitive to ionic strength and is maximal at $\mu = 0.05$. Identical results were obtained whether NaCl or KCl was used. Sucrose (0.32 M) is without effect. Virtually all specific binding activity (>95%) is sedimented at 20,000 × *g* in 10 min.

Specific binding is sensitive to trypsin and Pronase and to three different sulfhydryl reagents (Table 3). It is unaffected by phospholipase A and C as well as by sodium azide, sodium fluoride, and prostaglandins E_1 and E_2.

Competition Experiments. Table 4 shows competition experiments with several morphine-type drugs. ED_{50} is the concentration of drug that prevents 50% of the binding of [^{3}H]etorphine (3×10^{-9} M). The ED_{50} values generally correlate with the *in vivo* potency of agonists and antagonists, but the quantitative differences do not necessarily correspond. Thus, etorphine has been reported to be about 1000-times more active than levorphanol in rats (7), but the binding affinities differ by only 8-fold. Levorphanol and morphine, on the other hand, show similar differences (about 10-fold) in binding and in *in vivo* potency. Naloxone is 6-fold more effective than morphine in the prevention of [^{3}H]-etorphine binding, while it was reported to be less effective than morphine in preventing binding of naloxone (3). Dextrorphan exhibits an affinity $^1/_{40,000}$ that of its enantiomorph levorphanol.

DISCUSSION

The amount and many properties of the stereospecific binding of [^{3}H]etorphine are similar to those recently reported by Pert and Snyder for [^{3}H]naloxone (3). About 2 to 3×10^{-11}

TABLE 2. *Effect of ionic strength on specific binding of [^{3}H]etorphine*

Ionic strength (μ)	pmol Specifically bound	% of value at $\mu = 0.05$
0.01	0.48	86
0.05	0.56	100
0.10	0.35	64
0.15	0.25	46
0.20	0.19	35
0.50	0.07	13

Rat-brain homogenate (4.5 mg of protein per 2 ml) was incubated with [^{3}H]etorphine in 0.05 M Tris·HCl buffer (pH 7.4) with NaCl added to give the desired ionic strength. Similar results were obtained when KCl was used.

TABLE 3. *Effect of various reagents on specific binding of [^{3}H]etorphine to rat-brain homogenate*

Reagent	Concentration	% of Control*
N-Ethylmaleimide	10^{-4} M	60
	10^{-3} M	32
p-OH-mercuribenzoate	10^{-5} M	88
	10^{-4} M	0
Iodoacetamide	10^{-3} M	100
	10^{-2} M	47
Trypsin	1 mg	11
	10 mg	20
Pronase	6 μg	48
	60 μg	0
Phospholipase A	100 μg†	107
	200 μg†	100
Phospholipase C	50 μg	94
	100 μg	91
Sodium azide	4×10^{-4} M	90
	4×10^{-3} M	98
Sodium fluoride	2.5×10^{-2} M	90
Prostaglandin E_1	3×10^{-8} M	99
	3×10^{-7} M	100
Prostaglandin E_2	3×10^{-8} M	100
	3×10^{-7} M	100

* Reagents were added to 2 ml of brain homogenate (3–4.5 mg of protein) during preincubation with dextrorphan or levorphanol. The control samples bound stereospecifically an average of 0.5 pmol of [^{3}H]etorphine.

† Refers to the quantity of snake venom before heat denaturation.

TABLE 4. *Competition of drugs for specific binding of [^{3}H]etorphine to rat-brain homogenate*

Drug	ED_{50} (M)
Etorphine	3×10^{-9}
Levorphanol	2×10^{-8}
Naloxone	3×10^{-8}
Nalorphine	5×10^{-8}
Morphine	2×10^{-7}
D(−) Methadone	3×10^{-7}
Dextrorphan	8×10^{-4}

3×10^{-9} M [^{3}H]etorphine was used. The percent decrease of stereospecific binding was determined for 5 or 6 concentrations of each drug. The ED_{50} values were determined from probit-log plots of the data.

mol are bound per rat brain, about 100-times the quantity of opiate receptors calculated by V. P. Dole (8) and considerably lower than the [^{3}H]levorphanol binding reported by Goldstein *et al.* (2). Dole's calculations rest on assumptions regarding the brain concentration and specific binding of etorphine; moreover, all receptors need not be saturated at therapeutic amount of drug. The amount of specific binding observed is, therefore, in a reasonable range for opiate receptors.

The results presented provide evidence that stable binding can be observed with a morphine-like agonist of sufficiently high affinity. This constitutes important evidence towards establishing that the binding observed is due to opiate receptors. Moreover, these results do not support the possibility that agonists alter the receptors upon binding in a manner that reduces their affinity for agonist binding. It is not clear why we were unable to observe specific binding of dihydromorphine. Morphine (and presumably dihydromorphine) has an affinity $^1/_6$ that of naloxone and $^1/_{60}$ that of etorphine. This low affinity could be responsible for the lability of its binding, but more studies are required. The surprisingly small difference in affinity between etorphine and levorphanol suggests that affinity for receptors is only one of several factors responsible for the enormous potency of etorphine.

Two of our observations are at variance with those of Pert and Snyder for binding of [^{3}H]naloxone. Morphine is distinctly less effective than naloxone in preventing the binding of [^{3}H]etorphine, while the opposite appears to be true for binding of [^{3}H]naloxone. High ionic strength effectively prevents etorphine binding, while it appears not to affect that of naloxone. Should the latter difference be a general one between agonists and antagonists, it may reflect the difference in receptor binding that results in their distinct pharmacological properties.

The effects of proteolytic enzymes and sulfhydryl reagents indicate that protein is part of the receptor molecule and that a sulfhydryl group is located at or near the binding site. The lack of effect of phospholipases A and C may indicate that phospholipids do not have a direct role in the binding.

The absence of any effect by high concentrations of sodium azide and sodium fluoride suggest that the binding is not dependent on energy from oxidative metabolism or glycolysis, which provides evidence that we are, indeed, observing binding rather than active transport into a subcellular organelle.

Ehrenpreis and Greenberg (9) observed that prostaglandins antagonize the inhibition by morphine of electrically stimulated contraction of isolated guinea pig ileum, suggesting interaction at a common site. In our system no competition for [^{3}H]etorphine binding is observed with prostaglandins E_1 and E_2.

The reports from Snyder's and our laboratory lend strong support to the existence of specific opiate receptors restricted to nervous tissue. The existence of a sensitive binding assay should make possible rapid progress in the study and ultimate purification of these receptors.

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1. Van Praag, D. & Simon, E. J. (1966) *Proc. Soc. Exp. Biol. Med.* **122**, 6–11.
2. Goldstein, A., Lowney, L. I. & Pal, B. K. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 1742–1747.
3. Pert, C. B. & Snyder, S. H. (1973) *Science* **179**, 1011–1014.
4. Blecher, M. (1966) *Biochem. Biophys. Res. Commun.* **23**, 68–74.
5. Simon, E. J., Dole, W. P. & Hiller, J. M. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 1835–1837.
6. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
7. Blane, G. F., Boura, A. L. A., Fitzgerald, A. E. & Lister, R. E. (1967) *Brit. J. Pharmacol. Chemother.* **30**, 11–22.
8. Dole, V. P. (1970) *Annu. Rev. Biochem.* **39**, 821–840.
9. Ehrenpreis, S. & Greenberg, J. (1973) *Fed. Proc.* **32**, 788, Abstr.