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[^{3}H]Etorphine Receptor Binding *in Vivo*

Small Fractional Occupancy Elicits Analgesia

DAVID C. PERRY,[1] JAN S. ROSENBAUM, MICHAEL KUROWSKI, AND WOLFGANG SADÉE
University of California School of Pharmacy, San Francisco, California 94143

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SUMMARY

The cerebral receptor binding of [^{3}H]etorphine in intact rats was determined with the use of a membrane filtration method performed immediately after brain excision and homogenization. Maximal binding coincided with maximal analgesic response. The *in vivo* binding was stereospecific and saturable, with a total binding population of 15–20 pmoles/g of brain. Fractional occupancy of the labeled binding sites at analgesic etorphine doses was exceedingly small (2% at the ED_{50}, tail flick assay). A small analgesic receptor subpopulation with high affinity could account for this result; however, the binding increased linearly between doses of 0.1 and 10 μg/kg, and the subpopulation should have been saturated in this dosage range. Moreover, etorphine analgesia is reversed rapidly, whereas a high-affinity analgesic receptor should release bound etorphine rather slowly. The *in vivo* dissociation half-life of [^{3}H]etorphine from its binding sites was relatively fast (~50 sec); in contrast, following identical labeling procedures *in vivo*, the subsequent *in vitro* dissociation rates were much slower (in Tris buffer with or without NaCl) and similar to those previously reported for high-affinity etorphine binding sites. The rapid *in vivo* dissociation rate was largely restored *in vitro*, when both Na^+ and guanylyl imidodiphosphate (a hydrolysis-resistant GTP analogue) were added immediately after homogenization and dilution. These results suggest that the majority of the [^{3}H]etorphine binding sites *in vivo* are regulated by Na^+ and GTP and that etorphine causes analgesia at a low fractional occupancy of these sites. Such a relationship could result from a high receptor-effector coupling efficiency via GTP regulatory units. The occupancy *in vivo* of the [^{3}H]etorphine sites was also measured for the following series of opiates with increasing antagonistic properties (or decreasing agonistic efficacy): morphine, pentazocine, nalorphine, levallorphan, and diprenorphine. Agonistic effects required an increasing fractional occupancy within this series, whereas antagonistic effects, if present, were approximately proportional to the fractional occupancy. This result supports a receptor binding-effect model that is based on a low fractional receptor occupancy at analgesic doses of the pure agonist etorphine.

INTRODUCTION

Binding studies *in vitro* have been successfully employed to demonstrate the existence of specific opiate receptors (for review see ref. 1). The drug-receptor binding behavior in intact animals has been less extensively investigated. Several groups, notably Höllt and Wüster (1) and Höllt and Herz (2) have used indirect methods on the basis of tracer concentration ratios in various tissues to measure opiate agonist binding *in vivo*. A rapid filtration technique for directly measuring *in vivo* cerebral binding was introduced by Pert and Snyder (3) in a study of opiate antagonists. Previous attempts to demonstrate *in vivo* receptor binding of opiate agonists either failed (4) or yielded exceedingly low amounts of specifically bound tracer (5). In contrast, the opiate agonists possess a high receptor affinity *in vitro* in Tris buffer homogenates, and receptor affinity correlates well with agonist potency (6). However, agonist binding is greatly affected by sodium ions and guanine nucleotides, whereas the binding of antagonists is largely unchanged by these modulators (7, 8). It is therefore important to determine to what extent these factors regulate agonist binding *in vivo*.

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[1] Present address, Department of Neurosciences, Johns Hopkins University School of Medicine, Baltimore, Md.

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We report here the results of studies on the *in vivo* cerebral binding of the potent opiate agonist, [^{3}H]etorphine. This tracer is known to bind *in vitro* with similar affinity to the μ, δ, and the recently identified κ,σ sites of the cerebral opiate receptor system (e.g., refs. 9–11). Here, saturable stereospecific *in vivo* binding of etorphine in rat brain is demonstrated. Moreover, the opiate agonists etorphine and morphine exert their analgesic effects at a very low fractional occupancy of those receptor sites that are labeled by [^{3}H]etorphine. The results are consistent with a receptor binding model that includes receptor coupling to a physiological effector via a GTP-sensitive mechanism.

MATERIALS AND METHODS

Determination of [^{3}H]etorphine binding in vivo. Animals received s.c. injections of [^{3}H]etorphine tracer (20 μCi/kg for most experiments, unless otherwise noted) which was diluted to 0.5 ml with 0.9% NaCl; in some experiments, various amounts of unlabeled drug were included in this injection mixture (co-administration). Animals were decapitated after different time intervals, and their brains were rapidly (1.0 min) removed, the cerebellum was discarded, and the remainder was homogenized for 20 sec in 30 ml (20 volumes) of ice-cold 50 mM Tris buffer (pH 7.4 at 37°) in a 50-ml Erlenmeyer flask, using a Brinkman Polytron (PT-10st, Setting 6). In this medium at this temperature, no net association or dissociation of the specifically bound tracer was observable over a period of at least 60 min. Also, no variation in the total binding was detected when the time between decapitation and homogenization and the time of homogenization were varied. Aliquots of 0.5 or 1.0 ml were immediately filtered under low pressure through Whatman GF/B glass-fiber filters (2.4 cm) and rinsed twice with 8 ml of ice-cold buffer. Duplicate or triplicate filter samples were placed in scintillation vials, and 10 ml of Aquasol (New England Nuclear Corporation, Boston, Mass.) were added; samples were counted for 10 min by liquid scintillation (Beckman LS 9000) and the disintegrations per minute per milliliter of particulate-bound [^{3}H]etorphine were calculated using channels-ratio quench correction. For the total [^{3}H]etorphine in homogenate, 0.5-, 1.0-, and 1.5-ml aliquots of homogenate were counted directly in 10 ml of Aquasol and the disintegrations per minute per milliliter were calculated, thus allowing calculation of the percentage of [^{3}H]etorphine bound in brain.

Binding blank. Several experiments were performed to examine the specificity of the bound material being measured. Brains of untreated animals were homogenized in ice-cold Tris buffer with added [^{3}H]etorphine (about 25 nCi/brain, which is in the range of total brain levels found after *in vivo* labeling experiments), either with or without 10^{-6} M unlabeled etorphine. Bound and total radioactivity were determined following the procedure for *in vivo* bound tracer. In the presence of excess unlabeled competitor, 2% of the added radioactivity was retained on the filters (representing nonspecific binding to brain tissue and directly to the filters). This increased to 4% when the unlabeled etorphine was omitted, suggesting the possibility that 2% of the tracer not already specifically bound following *in vivo* labeling might redistribute to available specific binding sites during the approximately 40 sec of homogenization and filtration. Since about one-half of the total brain radioactivity is already specifically bound in most experiments, this would mean that in these cases 1% of the total brain radioactivity may redistribute from unbound to specifically bound, which would suggest a total blank value of about 3%.

This was confirmed by means of an *in vivo* binding blank. Five animals were given [^{3}H]etorphine (either 40 or 60 μCi/kg) along with a saturating dose of naloxone (10 mg/kg), diprenorphine (2 mg/kg), or levallorphan (80 mg/kg), which should prevent all specific [^{3}H]etorphine binding. These animals gave an average of 3.3% of the total brain radioactivity bound to filters. This value was subtracted as a blank for nonspecific binding in subsequent experiments.

Metabolism of [^{3}H]etorphine. Two animals were each given etorphine, 20 μCi/kg; one dose was given with only tracer and one with 5 μg/kg unlabeled drug. Both animals were killed at 20 min. The brains were homogenized and filtered as described, and the filtrate was collected. Filters were extracted in methanol for 24 hr with unlabeled etorphine (5 μg/ml) added as carrier. An aliquot of methanol was counted directly (as described above), and the remainder was evaporated and chromatographed by reverse-phase HPLC[2] (see below for conditions); fractions were collected and counted. Filtrate from each brain was combined, 100 μg of unlabled etorphine were added to 90 ml of filtrate, and the nature of the radioactivity in the filtrate was determined. To detect specifically unchanged [^{3}H]etorphine, 30 ml of filtrate were extracted with organic solvent (two times, 25 ml of ethyl acetate) at pH 9; the residue was chromatographed by reverse-phase HPLC and the fractions were collected and counted by liquid scintillation. To measure total ^{3}H-activity, 30 ml of filtrate were evaporated and analyzed directly. Greater than 95% of the ^{3}H-activity on the filters co-chromatographed with authentic etorphine, both following tracer alone and after a 5 μg/kg dose. In the filtrate, we found that between 85% and 95% of the radioactivity is chromatographically identical with etorphine, with 5–15% as more polar metabolic products. All of the total radioactivity measured directly in the homogenate was accounted for in the various chromatographic fractions. In conclusion, the ^{3}H-activity recovered on the membrane filters represents unchanged etorphine, whereas total ^{3}H-activity contains a small component (~8%) of etorphine metabolites.

Analgesia. Analgesia was determined using the tail flick method (12) after s.c. injection of etorphine. To achieve a uniform absorption of light, tails were first painted with black ink and allowed to dry. Control latencies were determined; animals with latencies outside the range 2.0–4.0 sec were discarded. The cutoff time was set at 10 sec; longer times were not used to prevent damage to the tail, and because the quantal nature of the response

[2] The abbreviations used are: HPLC, high-pressure liquid chromatography; GPP(NH)P, guanylyl imidodiphosphate.

meant that animals not flicking their tails at 10 sec would almost certainly not flick at some later time, such as 20 or 30 sec. For analgesia reversal studies, animals received injections of diphrenorphine (2 mg/kg) 17 min after various doses of etorphine, at which time peak receptor binding was reached, and tested for tail flick latency at 30-sec intervals. The time from antagonist injection to the first latency below 10 sec was defined as the "lag time." To determine the analgesic ED_{50}, animals were tested 20 min following s.c. injection of different doses. An ED_{50} of 0.9 μg/kg was obtained by log-probit analysis using the percentage of animals responding at each dose (three to six animals per dose).

In vitro dissociation of ^{3}H-tracer following in vivo labeling. [^{3}H]etorphine (15–60 μCi/kg) was administered s.c. in 0.9% NaCl solution; animals were decapitated after 20 min and the initial binding was determined as described above. Immediately thereafter, dissociation of bound tracer was initiated by further diluting the original cold brain homogenate 10-fold with 50 mM Tris buffer at 37° containing 10^{-6} M unlabeled etorphine, with or without 100 mM NaCl; this yielded a final dilution of brain tissue of 210:1. In some experiments, 25 μM GPP(NH)P was included in the original and dilute homogenate. Binding at different times thereafter was assessed by filtering 5- or 10-ml aliquots of this dilute homogenate, as well as measuring the total radioactivity in the homogenate as described above. Data were expressed as the percentage of the initial binding (initial binding is defined as the percentage bound before dilution).

Materials. All animals used were female Sprague-Dawley rats weighing 180–220 g (Simonsen, Gilroy, Calif.). [^{3}H]Etorphine was obtained from the National Institute on Drug Abuse (33 Ci/mmole) and purchased for Amersham (Arlington Heights, Ill.) (42 Ci/mmole). Radioactive purity of the tracer was monitored periodically by reverse-phase HPLC, under conditions described earlier for analysis of [^{3}H]morphine (13), using a mobile phase of 3:1 methanol/10 mM sodium phosphate buffer (pH 6.7). Unlabeled etorphine was used as a carrier. Radioactivity was measured by liquid scintillation counting, as described above. Tracers were purified with this same HPLC system whenever radioactive purity was below 90%.

GPP(NH)P was purchased from Boehringer Mannheim (Indianapolis, Ind.). Diprenorphine HCl, etorphine HCl, and nalorphine HCl were supplied by the National Institute on Drug Abuse. Several compounds were generously supplied to us: levallorphan tartrate and dextrallorphan HBr (Hoffmann-La Roche, Nutley, N. J.); naloxone HCl (Endo Laboratories, Garden City, N. Y.) and pentazocine HCl (Sterling-Winthrop, Rensselaer, N. Y.). Morphine sulfate was purchased from Mallinckrodt (St. Louis, Mo.). All weights are expressed as the free base.

RESULTS

Time course of etorphine disposition and effects. The disposition *in vivo* of etorphine was studied with 20 μCi/kg ^{3}H-tracer doses given s.c. (equivalent to 0.26 μg/kg); the rats were killed at various time intervals, and total and bound radioactivity values were determined in the brain (minus cerebellum) (Fig. 1). Total and bound radioactivity peaked between 10 and 20 min, declining gradually thereafter. In a previous investigation, Dobbs (14) found that both brain levels and analgesia peaked at about 20 min following injection of etorphine to female rats. Equilibrium of the ligand with its binding site(s) appears to occur rapidly, since the bound radioactivity had already reached the plateau level of about 50% of the total radioactivity in the homogenate within 5 min, the first time point measured. Percentage binding remained at this level at all time points measured. In subsequent experiments, the amount of the tracer dose specifically bound was found to vary from about 35–55% of the total radioactivity in the homogenate; however, variation within a single experiment was ±5% at most.

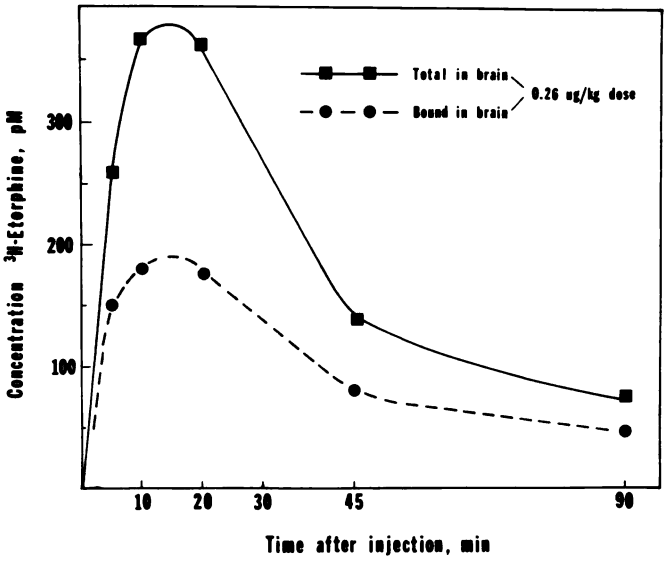

FIG. 1. *Time course of bound and total [^{3}H]etorphine in brain following a tracer dose*

The total amount and the amount bound per gram of brain tissue were measured as described at various times following s.c. injection of [^{3}H]etorphine (0.26 μg/kg). Each point represents the mean of determinations in two animals.

The time course of analgesia following a 1.5 μg/kg dose of etorphine was measured in two animals. Analgesia was maximal at 16 and 18 min, coinciding with the time of maximal [^{3}H]etorphine binding.

Stereospecificity. To determine the stereospecificity of [^{3}H]etorphine binding *in vivo*, [^{3}H]etorphine (40 μCi/kg) was administered either alone or together with levallorphan or dextrallorphan at different doses. Levallorphan decreased binding in a dose-dependent fashion, with an 80 mg/kg dose completely abolishing binding, whereas the pharmacologically inactive dextrallorphan had no effect on binding at doses of 0.8 and 80 mg/kg.

Saturability. To determine the saturability of [^{3}H]etorphine binding *in vivo*, [^{3}H]etorphine was given with increasing doses of unlabeled etorphine. Figure 2 shows the results as a saturation curve. Binding increased linearly with doses below 10 μg/kg (approximate dosage is indicated along the *top* of the graph). Linear regression analysis of bound versus total at lower doses (yielding total concentrations of 1 pmole/g of brain and below; see Fig. 2) produced a straight line with a slope of 0.452 (i.e., 45.2% bound; $r = 0.974$, $n = 29$). When the analysis was

extended to include brain concentrations of up to 10 pmoles/g (approximate dose 10 μg/kg), the slope of the linear regression line ($r = 0.987$, $n = 37$) was identical, indicating a lack of saturation within this range. The finding of 45% specific binding after [³H]etorphine tracer doses using the filtration method is similar to the results obtained by Höllt and Wüster (1) and Höllt and Herz (2) using the tissue radio method. The saturability of [³H] etorphine binding *in vivo* is demonstrated by the plateauing of the binding curve (Fig. 2); half-maximal binding is reached at ~50 pmoles/g of total brain level. The total brain levels of [³H]etorphine demonstrated no saturation and increased linearly throughout the entire dose range (0.2–200 μg/kg); approximately 0.35% of the dose is found in the brain 20 min after s.c. injection.

A fully saturating dose appears to occupy a receptor population of about 15–20 pmoles/g of brain. The etorphine results are presented as a displacement curve in Fig. 3; 50% reduction to control binding was reached at a dose of 50 μg/kg. This dose produced a total brain concentration of approximately 50 pmoles/g (50 nM); however, local free drug concentrations at receptor sites *in vivo* are unknown.

Correlation of receptor binding to etorphine analgesia. Tail flick analgesic tests were performed as described in order to correlate binding with pharmacological response. ED_{50} values for analgesia were estimated to be 0.8 μg/kg at 17 min (peak binding) and 0.9 μg/kg at 20 min. These values compare with those obtained s.c. by Blane *et al.* (15) in rats at 30 min [ED_{50} 1.5 μg/kg (tail pressure assay) and 0.76 μg/kg (antibradykinin assay)]. A dose of 0.9 μg/kg yielded a total brain level of about 1 pmole/g of brain, with ~0.4 pmole/g bound; thus at an ED_{50} dose only 2% of the total population of labeled binding sites was occupied. Fifty per cent occupancy occurred at a dose 50 times greater than the analgesic ED_{50}.

Displacement of bound [³H]etorphine by a series of opiate analgesics with increasing antagonistic potency. The following opiates were examined for their ability to inhibit the *in vivo* receptor binding of [³H]etorphine when coadministered with the tracer: morphine, pentazocine, nalorphine, levallorphan, and diprenorphine. These opiates are arranged in their order of increasing antagonist potency on the basis of the ratio of agonist over antagonist dosage ranges (Table 1; see also refs. 15–20). The results are given in Fig. 3, plotted on a log-probit scale to linearize the displacement curves.

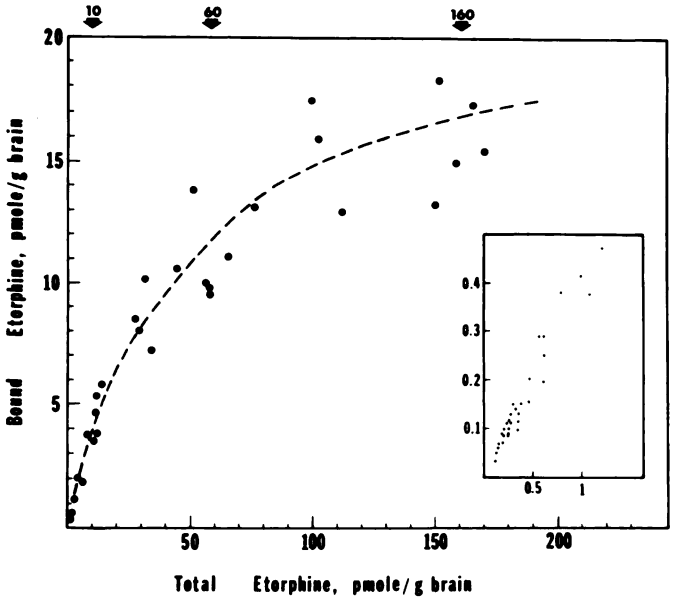

FIG. 2. *Saturation plot of bound versus total etorphine in brain 20 min after s.c. injection*

Each point represents the determination in an individual animal; *arrows* at *top* indicate approximate doses yielding these values (micrograms per kilogram). The *inset* shows the results at low concentrations obtained after doses of 1 μg/kg and below.

Not until doses of 100 and 200 mg/kg morphine was any significant depression of [³H]etorphine binding seen; 50% reduction in control binding of tracer by morphine occurred at a dose that is 50–900 times greater than reported values for the analgesic ED_{50} in rats (Table 1). Thus, etorphine and morphine, both of which are pure agonists, occupy a very small fraction of the receptors that are labeled by [³H]etorphine at analgesic doses. The

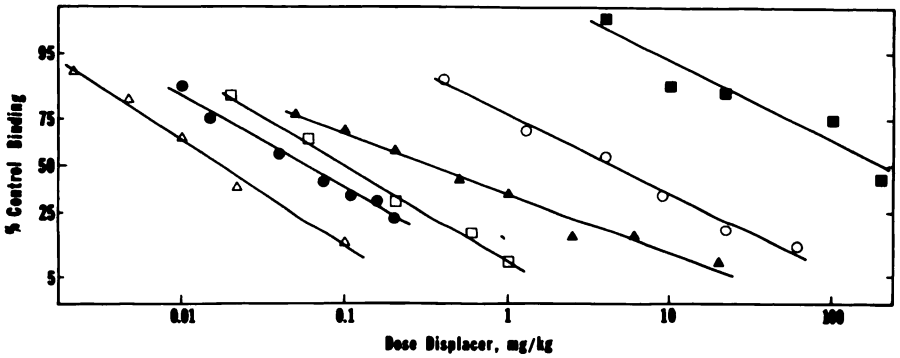

FIG. 3. *Inhibition of [³H]etorphine binding in vivo by various opiate displacers*

Unlabeled drug ("displacer") and [³H]etorphine tracer were coadministered by s.c. injection, and [³H]etorphine binding was measured as described 20 min later (morphine was injected 10 min before tracer). Control binding was measured in each experiment with each drug and was defined as the binding in animals administered tracer only ([³H]etorphine, 0.28 μg/kg). Each point is the mean of determinations in two to four animals, except for morphine (one animal per dose). Lines are linear regressions of the log dose versus probit of percentage of tracer bound (r = 0.95–0.99). The drugs used were morphine (■), pentazocine (○), nalorphine (▲), levallorphan (□), diprenorphine (△), and etorphine (●).

TABLE 1

Comparison of doses required to reduce [^{3}H]etorphine binding in vivo to 50% of control (ID_{50}), as determined by log-probit analysis (Fig. 3), to reported values for ED_{50} doses for analgesia and AD_{50} doses for inhibition of analgesia in rats

Doses are expressed as milligrams of free base per kilogram; administration was by s.c. route. Several different physiological tests for pain were employed, as noted.

Drug	ID_{50}	Analgesic ED_{50}			Antagonist AD_{50}	
Etorphine	0.057	0.00076[a] (15)	0.0009[b,c]	0.0015[b] (15)		
Morphine	180	0.2[b] (17)	1.1[a] (15)	2.5[e] (15)		
Pentazocine	4.2	0.95[d] (15)	1.85[a] (15)	5.5[f] (19)	1.2[e,g] (19)	3.9[b,h] (18)
					27[d,i] (15)	60[b,g] (15)
Nalorphine	0.32	0.20[d] (17)	3.2[a] (15)		0.13[b,h]	0.22[e,g] (19)
						0.38[e,g] (16)
Levallorphan	0.10	1.7[d] (17)	82[a] (18)		0.052[b,h] (18)	0.11[e,g] (19)
					0.20[e,g] (15)	0.24[a,e] (17)
Diprenorphine	0.016				0.004[e,g] (15)	0.008[e,g] (20)
					0.015[e,j] (16)	

[a] Antibradykinin assay.
[b] Tail flick assay.
[c] This study.
[d] Antiphenylquinone writhing assay.
[e] Tail pressure assay.
[f] Anti-NaCl abdominal constriction assay.
[g] Versus morphine analgesia.
[h] Versus meperidine analgesia.
[i] Versus oxymorphone analgesia.
[j] Versus nalorphine analgesia.

analgesic antagonists pentazocine, nalorphine, and levallorphan were capable of fully displacing [^{3}H]etorphine binding at high doses. The analgesic and antagonist dosage range is given in Table 1 along with the dose that displaced [^{3}H]etorphine by 50% (ID_{50}). Since analgesic dosages are dependent upon the quality and intensity of the nociceptive stimulus, exact correlations of binding and analgesia *in vivo* cannot be made; nevertheless, a general correlation can be made by comparing the *ranges* of analgesic dosages. Analgesic doses of the mixed opiates occupied an increasing fraction of the [^{3}H]etorphine receptor sites as the antagonistic character of the mixed opiate increased. The analgesic range of the strongly antagonistic levallorphan (17) required an occupancy of 90% and greater.

In contrast, displacement of [^{3}H]etorphine binding *in vivo* does appear to correlate with antagonist potency. The diprenorphine dose that displaced 50% of bound [^{3}H]etorphine (16 μg/kg) was close to the antagonistic ED_{50} (Table 1). Levallorphan behaves as an antagonist at lower doses, and its fractional binding corresponded well with its antagonist activity (Table 1). A similar comparison is obtained for nalorphine, although the analgesic and antagonist dosage range is less well-defined for this opiate.

[^{3}H]Etorphine receptor dissociation. In order to study the drug-receptor binding mechanism that is responsible for the low fractional occupancy at analgesic doses of agonists, the receptor dissociation kinetics of [^{3}H]etorphine was measured. To initiate dissociation *in vitro* after receptor labeling *in vivo*, the initial cold homogenate (1:20) was diluted to a final tissue dilution of 1:210 with Tris buffer at 37°, with 10^{-6} M unlabeled etorphine added to prevent any tracer reassociation; results are shown in Fig. 4 (■). A biphasic dissociation was observed; the initial phase accounted for about 25% of the bound material, with an apparent dissociation half-life of 9 min, whereas the remaining material dissociated more slowly ($t_{1/2}$ = 46 min). When these curves were feathered using curve-stripping techniques of biexponential decay curves, the initial phase was seen to have a $t_{1/2}$ of about 1 min. Previous investigations of [^{3}H]etorphine receptor kinet-

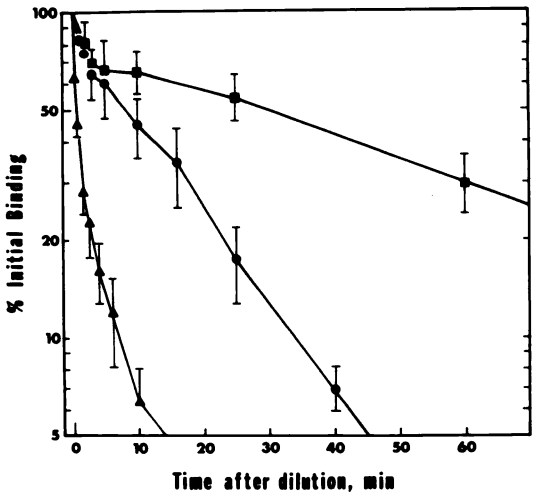

FIG. 4. *In vitro dissociation of [^{3}H]etorphine following in vivo labeling*

Dissociation was initiated after a 20-min *in vivo* labeling period with [^{3}H]etorphine (0.28 μg/kg) by a 210-fold dilution of brain tissue in 50 mM Tris buffer, pH 7 at 37°, with 10^{-6} M unlabeled etorphine added as displacer. Each point is the mean of determinations from three to nine animals; *error bars* represent standard deviation. Additions to the *in vitro* homogenate: none (■); 100 mM NaCl (●); 100 mM NaCl + 25 μM GPP(NH)P (▲). The brain was homogenized in ice-cold Tris buffer containing 25 μM GPP(NH)P, when dissociation was tested with NaCl plus GPP(NH)P.

ics at 37° following *in vitro* labeling have reported only the relatively long off-rates in the absence of sodium, i.e., $t_{1/2}$ = 34 min (21) and 25 min (8). Dissociation from neuroblastoma × glioma NG108-15 cell membranes at 32° also showed monophasic behavior, with a $t_{1/2}$ = 35 min (22).

To determine whether this initial rapid phase was a result of *in vivo* labeling rather than the conventional *in vitro* labeling, the actual *in vivo* dissociation was determined. After a 17-min prelabeling of cerebral sites *in vivo* by [³H]etorphine tracer, animals received s.c. injections of 2 mg/kg of the antagonist diprenorphine. With the use of [³H]diprenorphine tracer doses it was shown that this high dose rapidly enters the brain, approaching saturating levels within 30 sec; thus, the 2 mg/kg dose should serve as an effective *in vivo* chase. Animals were then killed at various times after antagonist chase administration, and the remaining [³H]etorphine binding was assessed by filtration immediately after homogenization (Fig. 5).

After an initial lag period, probably due to absorption, the binding can be seen to decrease very rapidly in a log-linear fashion, with a $t_{1/2}$ = 50 sec. A small fraction (<10%) appeared to dissociate more slowly. This fraction was difficult to analyze because of the low number of counts per minute, although it was definitely above background. However, the predominant fraction (90%) of the [³H]etorphine *in vivo* dissociated very rapidly.

We then examined whether the much slower dissociation kinetics of [³H]etorphine *in vitro* than in vivo was caused by a loss of regulatory factors that may normally be present *in vivo*, including sodium and guanine nucleotides (7, 8, 23). Figure 4 shows the *in vitro* dissociation of [³H]etorphine (after *in vivo* labeling) in the presence of

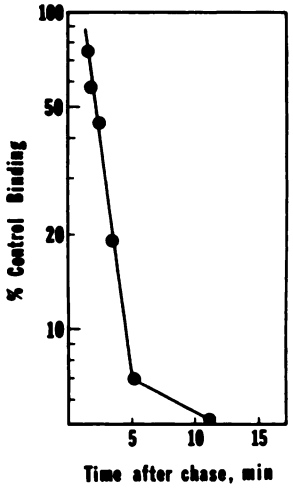

FIG. 5. *In vivo dissociation of bound [³H]etorphine after injection of antagonist chaser*

Diprenorphine (2 mg/kg) was injected as a chase 17 min following [³H]etorphine (40 μCi/kg); tracer binding was assessed at various times thereafter. Time represents total elapsed time between chase injection and brain homogenization (including approximately 1 min for brain removal during which time dissociation is likely to continue). Data are expressed as percentage of [³H]etorphine bound at 17 min without chaser (i.e., percentage of control binding). Each point is the mean of determinations in four animals.

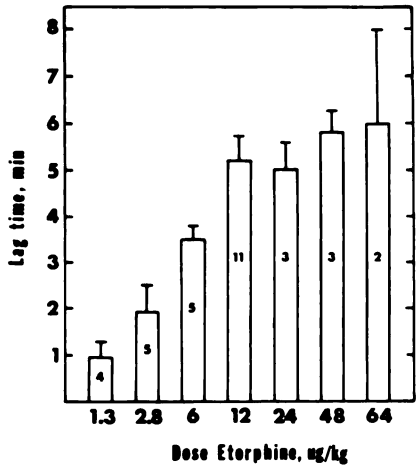

FIG. 6. *Lag time of analgesia reversal by diprenorphine (2 mg/kg) 17 min after various doses of etorphine*

Animals were tested for tail flick latency at 30-sec intervals following s.c. injection of the antagonist. Lag time is defined as the time between the injection of diprenorphine and the first latency below 10 sec (cutoff). *Error bars* represent standard error of the mean, except for 64 μg/kg, where the *bar* represents the range of two animals. *Numbers* within *bars* represent the number of animals tested at that dose. Lag times at different doses were compared by Student's *t*-test: 24 and 48 μg/kg did not differ significantly from 12 μg/kg, whereas all of the smaller doses differed from 12 μg/kg at a 95% confidence level or greater.

100 mM NaCl (●). Sodium significantly accelerated dissociation, but did not account for the difference between *in vitro* and *in vivo*. Dissociation was still biphasic; about 20% dissociated rapidly (with a $t_{1/2}$ of about 1 min), whereas the remainder had an off-rate of 11 min. Previous *in vitro* studies also found that dissociation of [³H]etorphine is accelerated in 100 mM NaCl and exhibits two phases (e.g., 8, 21, 22). When the brain was homogenized in Tris buffer containing 25 μM GPP(NH)P and incubated in the presence of sodium plus 25 μM GPP(NH)P, a nonhydrolyzable analogue of GTP, an even greater effect was seen (Fig. 4, ▲). The off-rate under these conditions was much faster, approaching that seen *in vivo*; about two-thirds of the tracer dissociated with a $t_{1/2}$ of less than 1 min, whereas the remaining slower phase had a $t_{1/2}$ of ~4 min. The addition of GPP(NH)P to the initial ice-cold Tris buffer brain homogenate was required to prevent a partial loss of the GPP(NH)P effect on [³H]etorphine off-rates during homogenization.[3]

Rate of etorphine analgesia reversal by diprenorphine. To ascertain how the dissociation of etorphine tracer *in vivo* correlates with the reversal of etorphine analgesia by antagonists, the rate of analgesia reversal was determined at maximally effective etorphine doses by a large dose of diprenophine (2 mg/kg at 17 min as used above for the *in vivo* tracer-receptor dissociation experiment). Maximally activating etorphine doses were determined by injecting increasing doses of etorphine and measuring the time from antagonist injection to the initial loss of analgesic response (i.e., "lag time"), using the tail flick method (12). The results of this experiment

[3] J. S. Rosenbaum, M. Kurowski, and W. Sadée, unpublished results.

are given in Fig. 6. The lag time increased with increasing doses of etorphine, until a plateau was reached at ~12 μg/kg. Higher doses did not prolong the lag time, although a dose of 12 μg/kg occupied only 20% of the [^{3}H] etorphine receptor sites. The maximal analgesia reversal lag time at 12 μg/kg etorphine was 5.2 min (±1.6 min SD, $n = 11$).

DISCUSSION

The data presented here provide evidence that etorphine and morphine analgesia in rats is associated with an exceedingly low fractional occupancy of those cerebral binding sites that are labeled by [^{3}H]etorphine *in vivo*. This finding suggests two mutually non-exclusive hypotheses: first, a small subpopulation of analgesic opiate receptors exists and, second, analgesia is elicited by a low fractional occupancy of the analgesic receptor population.

Receptor subpopulations and the effects of Na^+ and GPP(NH)P. In vitro binding studies have established the existence of at least three kinetically distinct binding site populations, i.e., μ, δ, and κ,σ (9–11). Etorphine was reported to bind to all three classes with similar affinity (in Tris buffer homogenates) (10). In rat brain, the capacities of these populations were calculated to be between 5 and 8 pmoles/g for each site (11). It is presently unknown which of these sites, if in fact they represent totally independent receptor systems, primarily mediates opiate agonist analgesia.

The total *in vivo* etorphine binding site population (15–20 pmole/g brain) was in the range of the sum of μ, δ, κ,σ sites demonstrated *in vitro* (11); moreover, dissociation curves in Tris buffer (with and without NaCl) following *in vivo* labeling were similar to those after *in vitro* labeling (8, 21, 22). These findings indicate that the *in vivo* [^{3}H]etorphine binding sites are to a large part identical with those labeled *in vitro*.

If only one of the demonstrated subpopulations were responsible for etorphine analgesia and if fractional occupancy were proportional to analgesic response, then this analgesic receptor subpopulation should have a much higher binding affinity for [^{3}H]etorphine *in vivo* in order to account for the low occupancy of all sites at analgesic doses. Furthermore, these receptor sites should saturate at doses of <10 μg/kg (Fig. 6). However, there was no evidence of high-affinity sites after low doses of etorphine (<10 μg/kg) (see *inset*, Fig. 2); rather, binding increased linearly within this dosage range, yet one cannot rule out the existence of a tiny subpopulation of analgesic receptors with binding capacities below those of the μ, δ, and κ,σ sites.

The *in vitro* binding affinity of pure opiate agonists is greatly reduced by Na^+ and GTP (7, 8, 21). However, the relative effect of these regulators on etorphine binding to the different opiate sites remains unknown. Reconstruction of the rapid *in vivo* [^{3}H]etorphine dissociation rate with the *in vitro* addition of Na^+ and GPP(NH)P (a hydrolysis-resistant GTP analogue) indicates that the major factors regulating *in vivo* opiate binding are indeed Na^+ and GTP.[4] This hypothesis is further supported by the relative potency of a series of opiates in displacing [^{3}H]-etorphine binding *in vivo* and *in vitro*. The following K_I values were reported *in vitro* (0.42 nM [^{3}H]etorphine, no added Na^+): etorphine 0.1 nM, diprenorphine 0.2 nM, levallorphan 0.3 nM, nalorphine 1.9 nM, morphine 8.9 nM, pentazocine 20 nM (24). When comparing this rank order with that obtained *in vivo*, the most notable change is the shift of the pure agonists etorphine and morphine to relatively lower affinity (Fig. 3). Delivery of the drug to the brain may play a role, but cannot explain these changes (compare with ^{3}H-tracer pharmacokinetics in refs. 13 and 25). Since the Na^+ and GTP effects are greater for pure agonists than for mixed agonists, these regulators are good candidates for mediating the *in vivo* changes of binding affinity.

In conclusion, the major portion of the *in vivo* [^{3}H] etorphine binding appears to be of relatively low affinity, possibly because of the effects of Na^+ and GTP; furthermore, no high-affinity subpopulation of the binding capacity of the μ, δ, and κ,σ sites is demonstrable which could be responsible for analgesia in proportion to its fractional occupancy. Finally, the rapid reversal of etorphine analgesia by diprenorphine (Fig. 6) argues against a small receptor subpopulation of high affinity that should release bound etorphine rather slowly.

Although these results do not positively rule out a small analgesic subpopulation, the concept of low fractional receptor occupancy at analgesic doses deserves further discussion. Low fractional occupancy may apply to either one of all of the binding sites populations shown *in vitro*.

Low fractional occupancy of the analgesic receptor sites. There is growing evidence that many pure agonists exert their maximal physiological effect at a low fractional occupancy of a variety of receptor systems (26–29). Moreover, an increasing number of receptor binding-effect models are being presented to account for such a relationship, e.g., (a) the "spare receptor" or "receptor reserve" hypothesis that invokes a greater number of receptor than effector molecules (26); (b) the receptor binding-effector coupling hypothesis that includes a loss of high-affinity binding of the agonist in the process of coupling (27); (c) the deglomeration-coupling model that assumes the existence of receptor oligomers and agonist activation by monomer formation (28); and (d) saturation of the physiological amplification cascade following activation of only a fraction of the total receptor population (29).

All of these models could accommodate the action of mixed agonist-antagonists, e.g., by assuming a reduced coupling efficiency or lower capacity to trigger deglomeration. In each case a higher fractional occupancy would be required for a given pharmacological effect. Therefore, a series of opiate agonists with increasing antagonist character was tested for fractional occupancy of the [^{3}H] etorphine binding sites *in vivo*. As expected on the basis of the concept of low fractional occupancy for pure

[4] Previous studies *in vitro* on the effects of Na^+ and GPP(NH)P on [^{3}H]etorphine binding kinetics (21, 22) have failed to find an off-rate approaching that observed here *in vivo*. A partial loss of GTP sensitivity of the opiate receptor system during *in vitro* preparation procedures was demonstrated in this laboratory.[3] The rapid *in vitro* off-rate in the presence of GPP(NH)P and Na^+ was only observable immediately after sacrifice using *in vivo* labeling; moreover, GPP(NH)P in the initial ice-cold medium was needed to preserve the large GPP(NH)P effect.

agonists, increasing antagonist character required increasing fractional occupancy of the [^{3}H]etorphine sites for analgesic activity (Fig. 3 and Table 1). The good agreement between antagonist ED_{50} of diprenorphine and levallorphan and the dose required to displace [^{3}H]etorphine by 50% satisfies the postulate that opiates with little or no agonist potency (or coupling efficiency) simply block receptor activation in proportion to their fractional occupancy. Similar findings for opiate antagonist binding *in vivo* were previously reported by Höllt and Wüster (1) and Höllt and Herz (2). Furthermore, the 50% reduction of *in vivo* [^{3}H]etorphine binding by the approximate ED_{50} of the relatively pure antagonist diprenorphine strengthens the hypothesis that the labeled sites are pharmacologically relevant.

Suggestive evidence in favor of a receptor binding effector coupling model as a basis for a low fractional occupancy at analgesic etorphine doses stems from the effects of Na^+ and GPP(NH)P on the *in vitro* dissociation rate. Both regulators are thought to be required for opiate agonist action (23) (presumably inhibition of adenylate cyclase). GTP is known to assist in the coupling of several neurotransmitter receptor systems to adenylate cyclase via a guanine nucleotide regulatory protein (28). Similar off-rates *in vivo* and *in vitro* in the presence of Na^+ and GPP(NH)P (under conditions that preserve maximal (GPP(NH)P sensitivity *in vitro*) strengthen this concept for the mode of action of etorphine *in vivo*. It is possible that the low fractional occupancy is based on triggering receptor-effector coupling via the GTP regulatory unit, followed by rapid agonist-receptor dissociation.

Profound changes in agonist-receptor binding *in vivo* also become evident when comparing the *in vivo* binding kinetics of the potent oripavine alkaloids etorphine (agonist) and diprenorphine (antagonist). Although their affinities *in vitro* are similar (0.1–0.2 nM), only [^{3}H]diprenorphine is selectively retained *in vivo* at cerebral binding sites over several hours (25). Furthermore, the apparent *in vivo* receptor dissociation of [^{3}H]diprenorphine after a chase dose of unlabeled diprenorphine is ~18 min (25), compared with only ~50 sec for [^{3}H]etorphine. This large difference in the *in vivo* dissociation rates could result from the inability of diprenorphine to trigger receptor-effector coupling with a subsequent reduction of binding affinity. Consistent with this hypothesis is the finding that antagonists are generally more effective than agonists in displacing [^{3}H]opiate tracers *in vivo*.

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REFERENCES

1. Höllt, J., and M. Wüster. The opiate receptors, in *Developments in Opiate Research* (A. Herz, ed.). Marcel Dekker, New York, 1–65 (1978).
2. Höllt, V., and A. Herz. *In vivo* receptor occupation by opiates and correlation to the pharmacological effect. *Fed. Proc.* **37**:158–161 (1978).
3. Pert, C. B., and S. H. Snyder. Identification of opiate receptor binding in intact animals. *Life Sci.* **16**:1623–1634 (1975).
4. Pert, C. B., S. H. Snyder, and M. J. Kuhar. Opiate receptor binding in intact animals, in *Tissue Responses to Addictive Drugs* (D. H. Ford and D. H. Clouet, eds.). Spectrum, New York, 89–101 (1976).
5. Mulé, S. J., G. Casella, and D. H. Clouet: The specificity of binding of the narcotic agonist etorphine in synaptic membranes of rat brain *in vivo*. *Psychopharmacologia* **44**:125–129 (1975).
6. Creese, I., and S. H. Snyder. Receptor binding and pharmacological activity of opiates in the guinea-pig intestine. *J. Pharmacol. Exp. Ther.* **194**:205–219 (1975).
7. Childers, S. R., and S. H. Snyder. Differential regulation by guanine nucleotides of opiate agonist and antagonist receptor interactions. *J. Neurochem.* **34**:583–593 (1980).
8. Simon, E. J., J. H. Hiller, J. Groth, and I. Edelman. Further properties of stereospecific opiate binding sites in rat brain: on the nature of the sodium effect. *J. Pharmacol. Exp. Ther.* **192**:531–537 (1975).
9. Chang, K.-J., E. Hazum, and P. Cuatrecasas. Novel opiate binding sites selective for benzomorphan drugs (enkephalins, morphine, diprenorphine, κ and σ receptors). *Proc. Natl. Acad. Sci. U. S. A.* **78**: 4141–4145 (1981).
10. Kosterlitz, H. W., and S. J. Paterson. Characterization of opioid receptors in nervous tissue. *Proc. R. Soc. Lond.* **210**:113–122 (1980).
11. Pfeiffer, A., and A. Herz. Demonstration and distribution of an opiate binding site in rat brain with high affinity for ethylketocyclazocine and SKF 10,047. *Biochim. Biophys. Acta* **101**:38–44 (1981).
12. D'Amour, F. E., and D. L. Smith. A method for determining loss of pain sensation. *J. Pharmacol. Exp. Ther.* **72**:74–79 (1941).
13. Mullis, K. B., D. C. Perry, A. M. Finn, B. Stafford, and W. Sadée. Morphine persistence in rat brain and serum after single doses. *J. Pharmacol. Exp. Ther.* **208**:228–231 (1979).
14. Dobbs, H. E. Effect of cyprenorphine (M285), a morphine antagonist, on the distribution and excretion of etorphine (M99), a potent morphine-like drug. *J. Pharmacol. Exp. Ther.* **160**:407–414 (1968).
15. Blane, G. F. Blockade of bradykinin-induced nociception in the rat as a test for analgesic drugs with particular reference to morphine antagonists. *J. Pharm. Pharmacol.* **19**:367–373 (1967).
16. Blane, G. F., and D. Dugdall. Interactions of narcotic antagonists and antagonist-analgesics. *J. Pharm. Pharmacol.* **20**:547–552 (1968).
17. Blumberg, H., P. S. Wolf, and H. B. Dayton. Use of writhing test for evaluating analgesic activity of narcotic antagonists. *Proc. Soc. Exp. Biol. Med.* **118**: 763–766 (1965).
18. Archer, S., N. F. Albertson, L. S. Harris, A. K. Pierson, and J. G. Bird. Pentazocine: strong analgesics and analgesic antagonists in the benzomorphan series. *J. Med. Chem.* **7**:123–127 (1964).
19. Collier, H. O. J., and C. Schneider. Some agonist-antagonist properties of morphine and antagonists in rodents, in *Agonist and Antagonist Actions of Narcotic Analgesic Drugs* (H. W. Kosterlitz, H. O. J. Collier, and J. E. Villareal, eds.). University Park Press, Baltimore, 128–135 (1973).
20. Lewis, J. W. Ring C-bridged derivatives of thebaine and oripavine. *Adv. Biochem. Psychopharmacol.* **8**:123–136 (1973).
21. Hambrook, J. M., and M. J. Rance. The interaction of buprenorphine with the opiate receptor: Lipophilicity as a determining factor in drug-receptor kinetics, in *Opiates and Endogenous Opioid Peptides* (H. W. Kosterlitz, ed.). Elsevier/North-Holland Biomedical Press, Amsterdam, 295–301 (1976).
22. Blume, A. J. Opiate binding to membrane preparations of neuroblastoma × glioma hybrid cells NG108-15: effects of ions and nucleotides. *Life Sci.* **22**: 1843–1852 (1978).
23. Blume, A. J., D. Lichtstein, and G. Boone. Coupling of opiate receptors to adenylate cyclase: Requirement for Na^+ and GTP. *Proc. Natl. Acad. Sci. U. S. A.* **76**:5626–5630 (1979).
24. Childers, S. R., I. Creese, A. M. Snowman, and S. H. Snyder. Opiate receptor binding affected differently by opiates and opioid peptides. *Eur. J. Pharmacol.* **55**:11–18 (1979).
25. Perry, D. C., K. B. Mullis, S. Øie, and W. Sadée. Opiate antagonist receptor binding *in vivo*: evidence for a new receptor binding model. *Brain Res.* **199**: 49–61 (1980).
26. Stephenson, R. P. A modification of receptor theory. *Br. J. Pharmacol.* **11**: 379–393 (1956).
27. Swillens, S., and J. E. Dumont. A unifying model of current concepts and data on adenylate cyclase activation of beta-adrenergic agonists. *Life Sci.* **27**: 1013–1028 (1980).
28. Rodbell, M. The role of hormone receptors and GTP-regulatory proteins in membrane transduction. *Nature (Lond.)* **284**:17–21 (1980).
29. Strickland, S., and J. N. Loeb. Obligatory separation of hormone binding and biological response curves in systems dependent upon secondary mediators of hormone action. *Proc. Natl. Acad. Sci. U. S. A.* **78**:1366–1370 (1981).

Send reprint requests to: Dr. Wolfgang Sadée, University of California School of Pharmacy, San Francisco, Calif. 94143.