*Proc. Natl. Acad. Sci. USA*
Vol. 89, pp. 10198–10202, November 1992
Neurobiology

# Guanine nucleotide-binding protein-coupled and -uncoupled states of opioid receptors and their relevance to the determination of subtypes

(arylacetamides/bremazocine/κ receptors)

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*Communicated by H. W. Kosterlitz June 3, 1992 (received for review March 28, 1991)*

ABSTRACT **Opioid receptors are currently classified as μ, δ, and κ types, but various subtypes have also been proposed. We have investigated whether subtypes exist by using [$^3$H]bremazocine. [$^3$H]Bremazocine binds to twice as many naloxone-sensitive sites as other nonselective opioid agonists, as shown in four membrane types that have very different ratios of μ, δ, and κ receptor types. [$^3$H]Bremazocine binding is completely inhibited by an excess (in unlabeled form) of other opioid ligands, with Hill coefficients of 0.8–0.95. These paradoxes can be explained if there are high- and low-affinity states of the μ, δ, and κ receptors and bremazocine binds with similar affinities to both states. We propose that these states are the guanine nucleotide-binding protein (G-protein)-coupled form and the uncoupled form of each receptor. As evidence for this proposal, the [$^3$H]bremazocine binding suffered little or no loss with G-protein-uncoupling treatments, whereas binding of other opioid agonists was fully sensitive. We conclude that [$^3$H]bremazocine offers a tool for the measurement of the total pools of coupled and uncoupled opioid receptors and that much of the previous characterization of opioid receptor subtypes reflects, instead, a significant pool of G-protein-uncoupled opioid receptors.**

The receptors for endogenous opioids are currently divided into three generally accepted types, namely, μ, δ, and κ. It is widely accepted that these receptors are members of the guanine nucleotide-binding protein (G protein)-linked superfamily and their responses show characteristics of being mediated by a second messenger system. Precise pharmacological characterization of the opioid receptor types was delayed until the synthesis and use of type-selective ligands such as [D-Ala$^2$,MePhe$^4$,Glycol$^5$]enkephalin (DAGO) [for μ (1)] and [D-Pen$^2$,D-Pen$^5$]enkephalin [for δ (2)]. Characterization of the κ type receptors was particularly difficult because, in radioligand binding studies, the κ type initially had to be measured as the residual binding of nonselective opiods after the μ and δ types were suppressed by μ- and δ-selective agonists (1). The nonselective radioligands most commonly used for this purpose were the benzomorphans bremazocine and ethylketocyclazocine (EKC). Pharmacological studies have indicated that bremazocine is an antagonist/partial agonist at κ sites, a partial agonist or antagonist at δ sites, and an antagonist at μ sites (3–8). More recently, κ binding sites have been measured by a $^3$H-labeled arylacetamide series of ligands that include the agonists 5α,7α,8β-(−)-*N*-methyl,*N*-[(7-(1-pyrrolidinyl)-1-oxaspiro(4,5)-dec-8-yl)]benzeneacetamide (U69593) (9) and (+)-*trans*-*N*-methyl,*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide hydrochloride (PD117302) (10). However, it has been found that these compounds recognize only a fraction of the binding sites defined, by the suppression method, as κ (9, 10). This and similar evidence has led to proposals for κ receptor subtypes (11–15), although differing definitions of these subtypes have been deduced.

The binding of agonists to the μ, δ, and κ opioid receptors is also highly sensitive to nonhydrolyzable GTP analogs such as guanosine 5′-[β,γ-imido]triphosphate p[NH]ppG, which (in the presence of $Na^+$) cause a great decrease in agonist affinity without changing the $B_{max}$ (16, 17). p[NH]ppG promotes the dissociation of receptor–G-protein–agonist complexes (18). Similarly, exposure of membrane preparations to a high pH can inactivate G proteins, leaving β-, $\alpha_1$-, and $\alpha_2$-adrenergic receptors intact but lacking high-affinity agonist binding (19, 20), and this effect has been shown to operate likewise for opioid receptor uncoupling (Y. H. Wong, C. D.-M., and E.A.B., unpublished data). We have used these methods to identify the states of the opioid receptor that are detected by [$^3$H]bremazocine but that are not labeled with high affinity by other opioid agonists.

## MATERIALS AND METHODS

**Cell Culture.** BS2 is a stable clonal cell line, originally derived by Nirenberg and coworkers (21) by the fusion of Chinese hamster brainstem neurons in primary culture with cells of a mouse neuroblastoma line (N18TG); BS2 was subcloned by M. R. Hanley (Medical Research Council), and the final clone used here was identified by its [$^3$H]EKC binding by A. Borsodi (Biological Research Center, Szeged, Hungary). The cells were scraped from 35 confluent 150-mm plates for use in each membrane preparation.

**Membrane Preparation and Radioligand Binding Assays.** A fraction enriched in microvilli membranes from human placenta was prepared as described (22). Membranes from adult rat (Sprague–Dawley) forebrain, male guinea pig (Dunkin–Hartley) cerebellum, and BS2 cells were as described (17). The binding assays were performed in triplicate in 10 mM *N*-tris(hydroxymethyl)-2-aminoethane sulfonic acid (Tes, pH 7.4) in the presence of 6 mM $Mg^{2+}$ and protease inhibitors for 1 h at 30°C (17); the $B_{max}$ for [$^3$H]bremazocine was measured in every set of assays on each tissue, for standardization. Nonspecific binding was measured in the presence of 10 μM naloxone. The data were analyzed by linear regression of Scatchard plots or, in competition studies, of Hill plots.

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Abbreviations: DADLE, [D-Ala$^2$,D-Leu$^5$]enkephalin; DAGO, [D-Ala$^2$, MePhe$^4$,Glycol$^5$]enkephalin; Pen, penicillamine; DPDPE, [D-Pen$^2$,D-Pen$^5$]enkephalin; DSLET, [D-Ser$^2$, Leu$^5$]enkephalin; EKC, ethylketocyclazocine; p[NH]ppG, guanosine 5′-[β,γ-imido]triphosphate; G protein, guanine nucleotide-binding protein.

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Nonsignificantly different ($P = 0.05$) values were found by nonlinear curve fitting (GRAPHPAD program). Results shown are the mean ± SEM.

Membranes that were to be pretreated at high pH were (in the absence of $Mg^{2+}$) diluted 1:20 with ice-cold 50 mM sodium phosphate (at pH 7.4 or 11.5) containing 1 mM EDTA. After incubation on ice for 45 min, an equal volume of 100 mM Tes (pH 7.4) was added. After centrifugation at 45,000 × *g* for 30 min at 4°C, the membranes were resuspended in 10 mM Tes (pH 7.4) for radioligand binding. Analysis of similarly treated guinea pig cerebellum membranes (to be reported elsewhere) showed the irreversible loss of 80% of the total guanine nucleotide binding sites and essentially 100% of the G proteins that could be ADP-ribosylated with pertussis toxin.

**Materials.** [$^3$H]Etorphine (50, 30, or 47 Ci/mmol; 1 Ci = 37 GBq) was from Amersham, and [$^3$H]bremazocine (27.8, 16.2, 18.5, or 17.3 Ci/mmol), [$^3$H]DAGO (59 Ci/mmol), $^3$H-labeled [D-Ser$^2$,Leu$^5$]enkephalin ([$^3$H]DSLET; 32 Ci/mmol), [$^3$H]EKC (30 or 15 Ci/mmol), and [$^3$H]U69593 (60 or 40 Ci/mmol) were from DuPont/NEN. PD117302 and U69593 were gifts from J. Hughes (Parke–Davis); bremazocine was a gift from D. Romer (Sandoz Pharmaceutical). Placentas were obtained within 30 min of delivery by cesarian section.

## RESULTS

Membranes were prepared from four tissues and their ligand binding activity was determined in Tes buffer in the absence of $Na^+$ and the presence of 6 mM $Mg^{2+}$, which optimizes $\mu$ and $\delta$ ligand binding (17, 23), compared to that in Tris·HCl buffer alone; in general, millimolar levels of $Mg^{2+}$ maximize receptor–G protein coupling (18).

**Human Placenta.** As found by others (24), the density of opioid binding sites was highly variable among various placentas ($B_{max}$, 0–200 fmol/mg); the affinity, however, for [$^3$H]bremazocine was much less variable (Table 1). Only placentas with a $B_{max}$ value of >100 fmol/mg were used and, when comparing the $B_{max}$ values of different radioligands, the same placenta was used. The nonselective opioid ligands [$^3$H]etorphine and [$^3$H]EKC bound to ≈50% of the bremazocine sites, as did the $\kappa$-selective ligand [$^3$H]U69593 (Fig. 1*A* and Table 1). Paradoxically, at first sight, [$^3$H]bremazocine binding was completely inhibited by each of these ligands as well as by PD117302, *trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide (U50488), and naloxone (Fig. 1*B*). The $\mu$-selective and $\delta$-preferring ligands DAGO and [D-Ala$^2$,D-Leu$^5$]enkephalin (DADLE) had no measurable effect upon bremazocine binding (Fig. 1*B*).

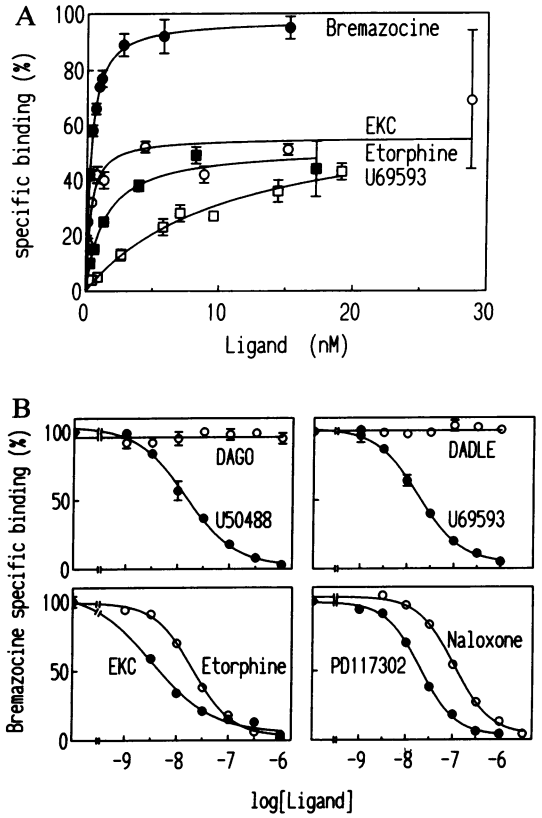


FIG. 1. Binding to opioid receptors in human placental membranes. The results shown here, and in Figs. 2 and 3, were obtained under steady-state conditions and are from an experiment representative of the number shown in the tables; the 100% level corresponds to 1000–2500 dpm measured. (*A*) Binding results are expressed as a percentage of the [$^3$H]bremazocine $B_{max}$ value for membranes from the same placenta. (*B*) Inhibition of [$^3$H]bremazocine (1.5 nM) binding with the compounds indicated.

Table 1. Radioligand binding parameters

| | $K_d$, nM | $B_{max}$, % of bremazocine $B_{max}$ | $n_H$ | $n$ |
|---|---|---|---|---|
| Placenta | | | | |
| Bremazocine | 0.40 ± 0.05 | 100 | 1.05 ± 0.01 | 7 |
| U69593 | 4.3 ± 1.2 | 50 ± 9 | 1.07 ± 0.07 | 2 |
| Etorphine | 1.1 ± 0.1 | 56 ± 8 | 0.98 ± 0.03 | 6 |
| EKC | 0.43 ± 0.1 | 52 ± 3 | 1.1 ± 0.1 | 3 |
| Rat forebrain | | | | |
| Bremazocine | 1.0 ± 0.1 | 100 | 0.98 ± 0.01 | 3 |
| EKC | 1.3 ± 0.2 | 44 ± 2 | 0.91 ± 0.30 | 4 |
| BS2 cells | | | | |
| Bremazocine | 2.0 ± 0.4 | 100 | 0.94 ± 0.03 | 8 |
| EKC | 2.2 ± 0.6 | 54 ± 9 | 0.94 ± 0.13 | 2 |
| Etorphine | 1.0 ± 0.5 | 45 ± 19 | 0.99 ± 0.09 | 2 |
| Guinea pig cerebellum | | | | |
| Bremazocine | 1.8 ± 0.3 | 100 | 0.96 ± 0.03 | 6 |
| EKC | 1.1 ± 0.1 | 45 ± 4 | 0.78 ± 0.05 | 3 |
| Etorphine | 1.5 ± 0.3 | 53 ± 11 | 0.96 ± 0.01 | 2 |
| U69593 | 3.9 ± 0.3 | 26 ± 1 | 0.90 ± 0.04 | 3 |
| DSLET | 1.9 ± 0.4 | 16 ± 1 | 0.97 ± 0.01 | 3 |
| DAGO | 1.7 ± 0.1 | 10 ± 1 | 0.90 ± 0.04 | 3 |

$n_H$, Hill coefficient; $n$, number of independent experiments. All of the $B_{max}$ values were significantly lower ($P < 0.01$) than the reference $B_{max}$ for [$^3$H]bremazocine.

Table 2. Ligand competition for [$^3$H]bremazocine binding

| | $K_i$, nM | $n_H$ | $n$ |
|---|---|---|---|
| Placenta | | | |
| U69593 | 5.0 ± 1.0 | 0.82 ± 0.04* | 3 |
| U50488 | 4.7 ± 0.1 | 0.89 ± 0.01* | 3 |
| PD117302 | 5.3 ± 0.4 | 0.82 ± 0.03* | 3 |
| Etorphine | 4.7 ± 0.3 | 0.92 ± 0.07 | 3 |
| EKC | 0.7 ± 0.1 | 0.92 ± 0.06 | 3 |
| Naloxone | 21 ± 3 | 0.94 ± 0.05 | 3 |
| DAGO | >1,000 | — | 3 |
| DADLE | >1,000 | — | 3 |
| Rat forebrain | | | |
| EKC | 1.6 ± 0.1 | 0.89 ± 0.06 | 3 |
| BS2 cells | | | |
| DADLE | 1.5, 5.5 | — | 2 |
| DPDPE | 1.8, 3.2 | — | 2 |
| Naloxone | 90, 110 | — | 2 |
| DAGO | 700, 900 | — | 2 |
| U50488 | 8,000, 10,000 | — | 2 |
| PD117302 | 5,000, 9,000 | — | 2 |
| Guinea pig cerebellum | | | |
| Etorphine | 2.8 ± 1.0 | 0.80 ± 0.06* | 3 |
| EKC | 2.1, 2.7 | 0.85, 0.79 | 2 |
| Naloxone | 90 ± 30 | 0.78 ± 0.10 | 3 |
| U50488 | — | 0.39 ± 0.01* | 3 |
| U69593 | — | 0.39 ± 0.02* | 3 |
| PD117302 | — | 0.43 ± 0.03* | 3 |

Reported $K_i$ values were calculated from $IC_{50}$ by the Cheng–Prusoff equation, except where $n_H$ was very low. No Hill coefficients were calculated for the BS2 data as insufficient data points were obtained between 10 and 90% inhibition.
*Significantly less than 1 ($P < 0.05$).

**Rat Forebrain.** A similar pattern of [$^3$H]bremazocine binding was observed in rat forebrain (Tables 1 and 2). [$^3$H]EKC bound to ≈50% of the [$^3$H]bremazocine binding sites (bremazocine $B_{max}$ = 530 ± 30 fmol/mg) but, again, unlabeled EKC was able to inhibit completely [$^3$H]bremazocine binding in competition studies (data not shown).

**BS2 Cells.** In membranes prepared from BS2 cells, [$^3$H]EKC and [$^3$H]etorphine again bound to ≈50% of the [$^3$H]bremazocine sites ($B_{max}$ = 360 ± 40 fmol/mg; Fig. 2*A* and Table 1). The bremazocine binding could be fully inhibited with high affinity by the δ-preferring DADLE and DPDPE (Fig. 2*B*) and also by naloxone (Table 2), whereas ligands selective for κ (U50488 and PD117302) and μ (DAGO) sites bound with far lower affinity (Fig. 2*B* and Table 2). The results suggest that virtually all opioid binding sites on these cells are of the δ type.

**Guinea Pig Cerebellum.** In membranes prepared from guinea pig cerebellum, [$^3$H]EKC and [$^3$H]etorphine likewise bound to ≈50% of the [$^3$H]bremazocine sites ($B_{max}$ = 230 ± 10 fmol/mg), and this 50% value was also equal to the sum of the sites recognized by the κ-, μ-, and δ-selective ligands [$^3$H]U69593, [$^3$H]DAGO, and [$^3$H]DSLET, respectively (Fig. 3*A* and Table 1). In competition studies (Fig. 3*B* and Table 2), EKC, etorphine, and naloxone again inhibited [$^3$H]bremazocine binding completely. DAGO and DADLE clearly showed polyphasic inhibition, whereas PD117302, U50488, and U69593 completely inhibited [$^3$H]bremazocine binding over a wide concentration range.

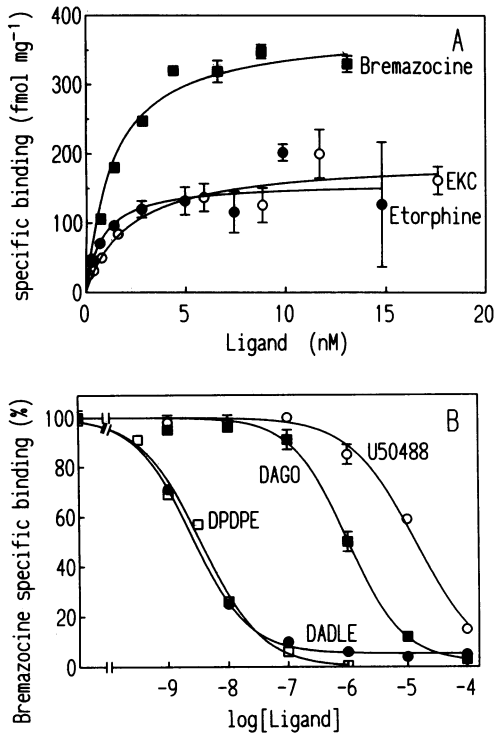


FIG. 2. Binding to opioid receptors in BS2 cell membranes. (*A*) Binding of the two radioligands indicated relative to that of [$^3$H]bremazocine. (*B*) Inhibition of [$^3$H]bremazocine (1.5 nM) binding. □, DPDPE; ●, DADLE; ■, DAGO; ○, U50488.

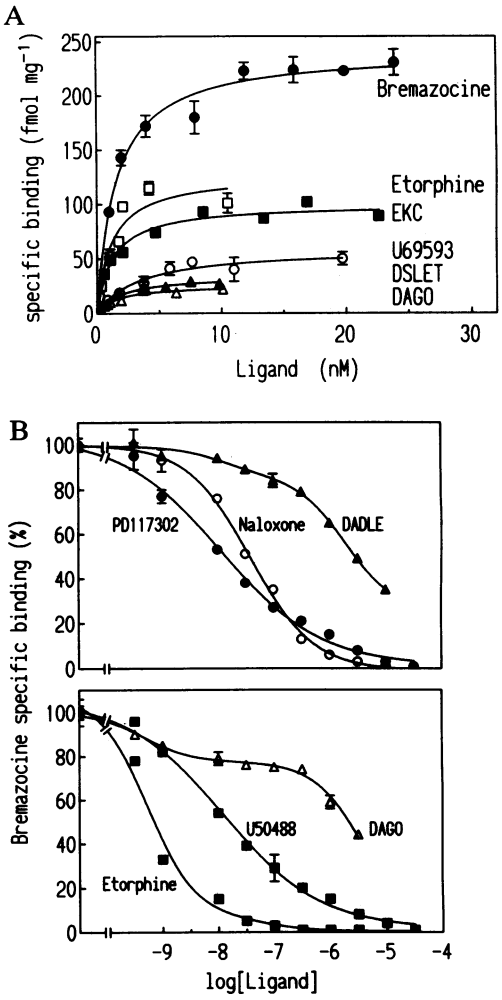


FIG. 3. Binding to opioid receptors in guinea pig cerebellum membranes. (*A*) Binding of the five radioligands indicated relative to that of [$^3$H]bremazocine. (*B*) Inhibition of [$^3$H]bremazocine (5 nM) binding with the compounds indicated.

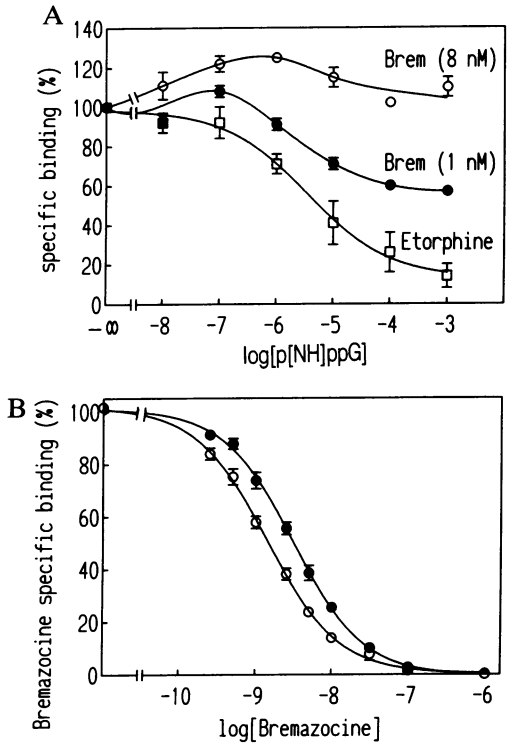


FIG. 4. (*A*) Inhibition of [$^3$H]bremazocine (1 nM or 8 nM; Brem) and [$^3$H]etorphine (1.7 nM) binding to opioid receptors in guinea pig cerebellum membranes by p[NH]ppG ($n$ = 3–5). The small initial increase may be due to a stimulation of the bremazocine antagonist action at $\mu$ sites at low concentrations of p[NH]ppG. (*B*) The inhibition (in those membranes) of [$^3$H]bremazocine (1 nM) binding by unlabeled bremazocine in the absence (○) or presence (●) of 0.1 mM p[NH]ppG/100 mM NaCl. The affinity of [$^3$H]bremazocine was reduced from 0.6 ± 0.2 nM to 1.9 ± 0.5 nM by the p[NH]ppG without significantly altering $B_{max}$ (210 ± 10 fmol/mg and 250 ± 40 fmol/mg, respectively).

**Effects of Guanine Nucleotide and of High pH Treatment.** p[NH]ppG [always used with NaCl at 100 mM for maximum effect (16, 17)] maximally inhibited 40% of the binding of [$^3$H]bremazocine (1 nM) to guinea pig cerebellum membranes (Fig. 4*A*). The residual binding could be completely inhibited by the $\kappa$-selective antagonist norbinaltorphimine, over a wide range [$10^{-10}$ to $10^{-5}$ M; Hill coefficient ($n_H$) = 0.36 ± 0.01; $IC_{50}$ = 3.5 ± 1.3 nM; $n$ = 3). However, at an 8-fold higher concentration of [$^3$H]bremazocine, the binding was not inhibited by p[NH]ppG. Likewise, there was only a small decrease in the [$^3$H]bremazocine affinity caused by a maximal concentration of p[NH]ppG (Fig. 4*B*). In contrast, the binding of [$^3$H]etorphine (1.7 nM, Fig. 4*A*) and [$^3$H]U69593, [$^3$H]DAGO, or [$^3$H]DSLET (all 6 nM, data not shown) was completely suppressed by 0.1 mM p[NH]ppG. GDP and adenosine 5′-[$\beta,\gamma$-imido]triphosphate (1 mM) did not inhibit [$^3$H]bremazocine binding. In placenta, BS2 cells, and rat forebrain, p[NH]ppG (0.1 mM) reduced the binding of [$^3$H]bremazocine (1 nM) to, respectively, 35 ± 4%, 40 ± 4%, and 72 ± 5% ($n$ = 3 in each case) of its value in the absence of added nucleotide.

An uncoupling pretreatment (19) of guinea pig cerebellum membranes at pH 11.5 reduced the specific binding of nonbenzomorphan agonists, when compared to control membranes maintained at pH 7.4 for the same period. This effect was always significantly greater (paired $t$ test; $P$ = 0.01) than for the [$^3$H]bremazocine binding. Thus, the fractions of the control binding in pH 11.5-treated reneutralized samples were as follows: [$^3$H]bremazocine (10 nM), 90 ± 8%; [$^3$H]U69593 (12 nM), 52 ± 14%; [$^3$H]DAGO (10 nM), 31 ± 9%; and [$^3$H]DSLET (6 nM), 51 ± 7% ($n$ = 5 in each case).

## DISCUSSION

In membranes prepared from tissues expressing entirely or mainly the $\kappa$ (placenta) (25), the $\delta$ (BS2 cells, this study), or the $\mu$ plus $\delta$ (rat forebrain) (26) types of the opioid receptor, the $B_{max}$ values (Table 1) show that bremazocine always binds to approximately twice the number of sites recognized by other nonselective opioid ligands, such as etorphine and EKC. Thus, this phenomenon is not restricted to one opioid type. The unidentified bremazocine binding sites appear to be opioid binding sites, as shown by their full sensitivity in competition studies to naloxone and other opioid ligands pharmacologically appropriate to the tissue used (Figs. 1–3). The latter observation appears paradoxical: a ligand such as EKC, or U69593 in the case of the pure $\kappa$ sites of the placenta, binds to only half of the bremazocine sites in direct binding studies but is able to displace bremazocine from all of them, without obvious biphasic behavior. A trivial explanation of this paradox would be that the quoted specific activity of the [$^3$H]bremazocine was seriously in error, the true value being 2-fold higher. However, the same phenomenon was found with four batches of [$^3$H]bremazocine, each with a different specific activity that was confirmed by both the manufacturer and ourselves.

This paradox may be explained most simply by taking into account the relative insensitivity of competition studies. Consider two equally abundant binding sites showing equal affinities ($K_d$, 1 nM) for a radioligand, L*, but with 10-fold different affinities for a competitor (say 5 and 50 nM). It may be calculated (27) that at equilibrium >90% of the binding of L* is inhibited by a competitor at 1 $\mu$M, with a Hill coefficient of 0.78, approaching the values observed here with EKC and etorphine (Table 2). Despite this, in a saturation binding experiment testing up to the 20 nM concentration used here, the radiolabeled competitor appears to recognize only a proportion (≈50%) of the sites labeled by L*. This difference is due to the higher ligand concentrations necessarily employed in competition experiments, which are inaccessible in direct binding studies. Such an explanation can cover the present case. It should be emphasized that the evidence for two binding sites comes directly from the double extent of labeling seen with [$^3$H]bremazocine compared to that with the other ligands. In principle, computer-based nonlinear curve fitting might detect the second site, but when applied (to the placental data), a two-site model was not preferred with statistical significance. We conclude that two bremazocine sites are present that have a similar affinity for bremazocine but different affinities for other nonselective opioid ligands.

In confirmation, our results with p[NH]ppG show that in all four tissues [$^3$H]bremazocine binds to two high-affinity components, a guanine-nucleotide-sensitive site and a guanine-nucleotide-insensitive site, whereas the other agonists tested (etorphine and $\mu$-, $\delta$-, and $\kappa$-selective agonists) bind with high affinity to only the guanine-nucleotide-sensitive site. The second [$^3$H]bremazocine affinity can, in fact, be measured separately by this device (Fig. 4*B*) and was found to have a $K_d$ value of 1.9 nM. The obvious interpretation is that the p[NH]ppG-sensitive and -insensitive sites represent the G-protein-coupled and -uncoupled states of the receptor. At the higher (8 nM) concentration, [$^3$H]bremazocine bound well to both the states so that a saturating level of p[NH]ppG had no effect upon the observed binding (Fig. 4*A*). At a sufficiently low concentration of [$^3$H]bremazocine (1 nM), binding in the absence of nucleotide was largely to the higher-affinity G-protein-coupled state. When the uncoupling was then maximized, an intermediate binding level was

attained. The residual binding apparently did not arise from bremazocine acting as an antagonist at $\mu$ and $\delta$ sites (hence being insensitive there to p[NH]ppG inhibition), since it occurs likewise in the placenta in the absence of $\mu$ and $\delta$ sites. Further, the residual binding (in guinea pig cerebellum) was inhibited by the $\kappa$-selective antagonist norbinaltorphimine with high affinity. The percentage inhibition of bremazocine binding by p[NH]ppG was much smaller in rat brain than in BS2 or placental membranes: this is understandable, since in rat brain membranes it is largely the $\delta$ site that is subject to such inhibition, because $\kappa$ forms only a small proportion of the opioid receptors there (26) and bremazocine is an antagonist at the $\mu$ receptor (7, 8).

Our interpretation is also supported by the insensitivity of [$^3$H]bremazocine binding to the high pH uncoupling pretreatment, compared to the sensitivity with other agonists. An alternative interpretation of the data is that the "excess" [$^3$H]bremazocine binding represents another opioid binding site, distinct from classical $\mu$, $\delta$, or $\kappa$ receptors. We reject this hypothesis since, under our experimental conditions, the same bremazocine/EKC binding ratio was observed in four tissue types with very different proportions of $\mu$, $\delta$, and $\kappa$ opioid receptors. We are unaware of any other independent receptor subtypes that are always equally abundant in such different tissues and we conclude that these extra sites are the G-protein-uncoupled states of the opioid receptors. The observation that the ratio of coupled to uncoupled receptors is 1:1 in all the tissues examined here may indicate some constraint on receptor–G-protein coupling, perhaps due to oligomeric opioid receptors (17) binding one G-protein per two opioid-binding subunits. The ability of bremazocine to bind with similar affinities to both the coupled and uncoupled states of the receptor may explain the low efficacy of this compound (4, 5). Agonist efficacy has been correlated with the ratio of affinities at analogous states of the muscarinic and $\beta$-adrenergic receptors (28, 29).

Our interpretation of opioid receptor ligand binding could explain many of the deductions of apparent $\kappa$ receptor multiplicity that have been made (for example, see refs. 11–15). These are mostly based upon observations that the binding of nonselective opioid radioligands, in the presence of agents that block binding at $\mu$ and $\delta$ sites, can be divided into sites that are sensitive and insensitive to various $\kappa$ agonists. When autoradiography was used, local variations in residual endogenous ligands and reagent impermeability could account for aberrant heterogeneity. In some membrane studies (13, 14), the relative abundances and properties of two classes of sites that have been seen by others are consistent with our data. In other cases, very different conditions of temperature, medium composition, and higher radioligand concentration than we used were applied: some uncoupled $\kappa$ receptors may also be labeled then where we (using, e.g., [$^3$H]EKC) label only the coupled state. In any case, our observations show that this method is likely to show apparent $\kappa$ receptor multiplicity even when only $\mu$, $\delta$, and $\kappa$ opioid types are present. (*i*) The ligands used to block binding at $\mu$ and $\delta$ sites are generally agonists, so that they will bind with higher affinity to the coupled than to the uncoupled states of $\mu$ and $\delta$ sites. This may leave radioligand binding at the uncoupled $\mu$ and $\delta$ sites largely unsuppressed. (*ii*) Since the compounds used to label the $\kappa$ site(s) (e.g., U69593) are also agonists, they, too, will show strongly differential affinity for the coupled and the uncoupled states; apparent heterogeneity may be observed even if all binding at $\mu$ and $\delta$ sites was suppressed. (*iii*) It has been pointed out (30) that several states of a single receptor type may exist, due to G-protein equilibria, that have different agonist affinities, further complicating interpretation of such data. Hence, it is not at all surprising that an apparent heterogeneity of $\kappa$ receptors is observed, but this need not have a relation to the true subtypes present. An analogous situation occurred with dopamine receptors (31), when the putative receptor subtypes identified were shown to be G-protein-coupled and -uncoupled states of the same receptors. Those putative subtypes did not correspond to the actual subtypes now known from DNA cloning; the same situation may well apply to the actual subtypes of the $\kappa$ receptor when cloned.

In conclusion, we find that the $\kappa$ receptor in its coupled state can at present best be recognized by the high-affinity binding of specific [$^3$H]arylacetamides and that the coupled and uncoupled states of $\mu$, $\delta$, and $\kappa$ receptors can be measured with [$^3$H]bremazocine. In general, we argue that it is wiser not to define subtypes for any of the G-protein-linked receptors by competition binding alone, due to the multiple receptor–G-protein equilibria involved.

We thank Dr. M. Nirenberg (National Institutes of Health) for BS2 cells, Drs. B. Morris (Medical Research Council) and M. Young (Department of Pharmacology, University of Cambridge) for helpful discussion, and Drs. C. McEwen and R. Young (DuPont/NEN) for mass spectroscopic reanalysis of the bremazocine batches. A.R. was supported by a Medical Research Council Partnership grant in association with ICI Pharmaceuticals.

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