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Solubilization and characterization of μ, δ, and κ opioid binding sites from guinea pig brain: Physical separation of κ receptors

(opioid receptor types/digitonin/sucrose density gradient)

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ABSTRACT **Sucrose density gradient centrifugation of digitonin-solubilized opioid binding sites from guinea pig brain and cerebellum was carried out. Centrifugation of extracts of whole brain into a gradient devoid of sodium and low in digitonin revealed the presence of two well-separated peaks of opioid binding activity. Peak A was shown to have the binding characteristics of κ sites, whereas peak B seems to be a mixture of μ and δ sites. When extracts of guinea pig cerebellum were treated in the same manner, a single peak of binding activity was obtained that coincided with peak A from guinea pig brain and exhibited the characteristics of κ binding sites. All three sites closely resemble their membrane-bound counterparts, retaining good affinity and selectivity for their appropriate ligands. The apparent sedimentation coefficients ($s_{20,w}$) of the digitonin-solubilized binding sites present in the two peaks are 19 *s* for peak A and 34–39 *s* for peak B, and the estimated apparent molecular weights are 400,000 for κ sites and 750,000–875,000 for the mixture of μ and δ sites. Our results suggest that κ sites constitute separate molecular entities from μ and δ sites.**

The existence of multiple types of opioid receptors has been suggested by pharmacological and biochemical studies. Three types of opioid receptors, μ (morphine), κ (ketocyclazocine), and σ (*N*-allylnormetazocine) were first postulated by Martin *et al.* (1, 2) on the basis of pharmacological studies on chronic spinal dogs. Kosterlitz's laboratory (3) has provided evidence to support the view that the enkephalins display preferential high affinity for yet another type of binding site, which they named δ. Since then, considerable neuropharmacological (3, 4) and biochemical (5–9) evidence has accumulated supporting the existence of distinct μ and δ binding sites.

It proved more difficult to provide biochemical evidence for the existence of κ and σ opioid receptors because of the lack of selective ligands and of tissues enriched in these receptor types. κ-type binding sites have now been demonstrated by studies of benzomorphan binding in the presence of μ and δ blockers in guinea pig brain (10). These studies were confirmed by James *et al.* (11) by means of inactivation of μ and δ sites by β-chlornaltrexamine in the presence of a selective κ ligand (dynorphin) to protect κ sites. There is now biochemical (12–15) and behavioral (16, 17) evidence suggesting that the binding sites for phencyclidine may be the σ opioid receptors.

Although the evidence for opioid receptor heterogeneity is quite convincing, little is known about the molecular basis of this heterogeneity. Solubilization and attempted separation and purification of receptor types are important steps toward this goal.

Results of early studies from this laboratory demonstrated that a [^{3}H]etorphine macromolecular complex could be solubilized by the nonionic detergent Brij 36T from prelabeled rat brain membranes (18). However, the solubilization of active opioid binding sites by this detergent could not be shown. More recently, we were able to demonstrate the solubilization in good yield of active opioid binding sites from toad brain (19, 20) and mammalian brain (21) using digitonin. Workers in other laboratories have solubilized active opioid binding sites from mammalian brain sources, using the cholic acid derivative 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (22) or Triton X-100 (23). In CHAPS-solubilized extracts from rat brain membranes, binding of δ opioid ligands was undetectable, although specific binding of μ and κ ligands was reported (24). In another recent study, solubilization of opioid binding sites by sonication and detergent treatment of rat brain membranes has been reported (25). In this extract, binding of μ, δ, and κ ligands at affinities considerably lower than those seen in membranes could be detected only after incorporation of lipids into the soluble material.

In our laboratory, good yields (30%–40%) of ^{3}H-labeled opiate antagonist binding to solubilized binding sites from mammalian brain were achieved by addition of NaCl during solubilization and by detergent dilution prior to binding (21). Under these optimal conditions, only very low levels of ^{3}H-labeled opiate agonist binding could be detected (26). This was found to be the result of the high susceptibility of opioid agonist binding to the inhibitory effect of even low levels of digitonin (0.01%–0.1%). Antagonist binding, however, was found to be protected by NaCl against inhibition by digitonin (27). We have recently found that, after fractionation of the soluble opioid binding sites from mammalian brain on a sucrose gradient containing no sodium and a lower concentration of digitonin, we were able to obtain good yields of ^{3}H-labeled opioid agonist binding to the soluble material (25%–35% yield based on the binding activity in comparable amounts of membrane preparation) (28). In this preliminary study (28), the method also allowed us to achieve separation of the binding activity of [^{3}H]bremazocine in the presence of μ and δ blockers from [^{3}H][D-Ala^2-MePhe^4-Gly-ol]enkephalin [^{3}H]DAMGE and [^{3}H][D-Ala^2-D-Leu^5]enkephalin [^{3}H]DADLE binding activities.

We now present further evidence for the physical separation of two peaks of binding activity and provide characterization of these peaks that strongly suggests that κ binding sites have been separated from a mixture of μ and δ sites.

MATERIALS AND METHODS

Male guinea pigs and male Sprague–Dawley rats were purchased from Taconic Farms (Germantown, NY). Whole brain minus cerebellum (which, for brevity, will be called

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Abbreviations: DAMGE, [D-Ala^2-MePhe^4-Gly-ol]enkephalin; DADLE, [D-Ala^2-D-Leu^5]enkephalin; DSLTE, [L-Ser^2-Leu^5-Thr^6]enkephalin; EKC, ethylketocyclazocine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

brain) of either species and the cerebellum of guinea pig were prepared essentially as described (20). Membrane preparations (1:6, wt/vol) were stored in 0.32 M sucrose at −70°C until needed. Membrane binding experiments were carried out as described (29).

Solubilization of guinea pig brain or cerebellum membranes was carried out as described (20, 21). In brief, particulate preparations (9–11 mg of protein per ml) were thawed and further diluted 1:4 (vol/vol) with 0.05 M Tris·HCl/1 mM dipotassium EDTA, pH 7.4/0.5% digitonin/0.1 mM leupeptin/1.0 M NaCl, final concentrations. The suspension was shaken for 30 min at 4°C followed by centrifugation at 100,000 × *g* for 35 min at 4°C. The clear supernatant was removed and used for further assays.

Continuous sucrose density gradients 3%–30% (10 ml) were prepared in Tris·HCl/EDTA/0.02% digitonin. Aliquots (1 ml) of solubilized material were layered on the top of the gradients and centrifuged at 100,000 × *g* for 2.5 hr at 4°C in a Beckman SW 41 Ti rotor. Eleven fractions (1 ml each) were collected with a Densi-Flow capillary pump. For binding assays, 0.3-ml aliquots of each fraction were diluted to 1 ml with Tris·HCl/EDTA buffer. Triplicate samples were incubated with [^{3}H]bremazocine in the absence and presence of 5 μM unlabeled bremazocine or diprenorphine for 60 min at 25°C. After incubation, 13% polyethylene glycol (Carbowax 6000) and 0.15% IgG (final concentrations) were added to the reaction mixture, according to the method of Cuatrecasas (30). Separation of free radiolabeled ligand from receptor–ligand complex was achieved either by filtration through GF/B Whatman filters, or by centrifugation at 8000 × *g* for 5 min, discarding the supernatant, and determining the radioactivity in the pellets. Specific binding in the fractionated soluble material as determined by the filtration method was 50%–60%, and by the centrifugation method it was 70%–75%.

Fractionation of soluble material that had been bound with ^{3}H-labeled opioids [bremazocine, ethylketocyclazocine (EKC), or diprenorphine] just prior to sucrose density centrifugation, was carried out as follows: 0.3-ml aliquots of the soluble material were diluted to 1 ml with Tris·HCl buffer (pH 7.4) and bound with 3 nM ^{3}H-labeled opioid ligand in the absence and presence of a 1000-fold excess of unlabeled ligand for 45 min at 25°C. These samples (1 ml) were then layered on separate sucrose gradients and fractionated as described above. To determine specific binding in the fractionated prebound soluble material, the radioactivity present in 0.5-ml aliquots from each fraction was determined.

The yield of opioid-binding activity in the solubilized material was calculated as follows: [specific binding to the soluble material/specific binding to membrane fraction] × 100. Specific binding was assayed at the same concentration of radioligand under identical conditions in membranes and solubilized preparations and derived from equal amounts of tissue.

Protein concentrations for all samples were determined by a modified Coomassie blue assay (31).

[^{3}H]Bremazocine (32.3 Ci/mmol; 1 Ci = 37 GBq) and [^{3}H]DADLE (43.6 Ci/mmol) were purchased from New England Nuclear and [^{3}H]DAMGE (49 Ci/mmol) was from Amersham. [^{3}H]Etorphine (22 Ci/mmol) and [^{3}H]EKC (35 Ci/mmol) were supplied by R. Hawks (National Institute on Drug Abuse). The following unlabeled ligands were used: (*d*,*l*)bremazocine (provided by D. Römer, Sandoz Pharmaceutical, Basel, Switzerland), DADLE (Peninsula Laboratories, San Carlos, CA), DAMGE (Reckitt and Coleman, Hull, England), EKC (Sterling Winthrop Research Institute, Rensselaer, NY), [D-Ser^2-Leu^5-Thr^6]enkephalin (DSLTE) donated by Bernard Roques. Other chemicals used were digitonin (ICN), polyethylene glycol 6000, *N*-ethylmaleimide, and trypsin (Sigma).

RESULTS

Since the proportion of κ, μ, and δ membrane-bound opioid receptors in the guinea pig brain has been reported to be approximately 4:3:3 (4), we have chosen this tissue as the starting material for the solubilization of the three major types of opioid binding sites. In addition, a recent report by Maurer *et al.* (32) indicates that 80%–90% of the opioid binding sites in the guinea pig cerebellum correspond to κ sites. In agreement with this study, we have found (Table 1) that specific binding of μ ([^{3}H]DAMGE) and δ-preferring ([^{3}H]DADLE) agonists to membranes of guinea pig cerebellum represents only 4% ± 1% of the binding seen with an equal amount of tissue from guinea pig brain. However, binding of [^{3}H]bremazocine, EKC, and etorphine [all of which display high affinities to κ, μ, and δ sites (10)] to cerebellum represents 55% ± 5% of the binding observed with brain preparations (Table 1). In addition, whereas the binding of these universal ligands to brain preparations in the presence of DAMGE and DADLE (0.1 μM each) was decreased by 50%–60%, the same concentrations of DAMGE and DADLE resulted in only 11%–15% decrease in the binding of bremazocine, EKC, and etorphine to guinea pig cerebellar membranes (Table 1).

The ultracentrifugal supernatants of digitonin-solubilized membranes derived from guinea pig brain or cerebellum were assayed for [^{3}H]bremazocine specific binding. The specific binding recovered in both soluble preparations represented 25%–30% of the binding present in the respective membrane preparations.

Fractionation of the extracts was conducted by using a continuous 3%–30% sucrose density gradient containing 0.02% digitonin. The length of time that the gradient is exposed to centrifugation is quite crucial. Centrifugation for 2.5–3 hr (at 100,000 × *g*) allowed migration of the applied soluble macromolecules into discrete areas of the gradient and good recovery of specific ^{3}H-labeled opioid binding to the fractionated material. Prolonged centrifugation (5–15 hr) resulted in a 40%–60% decrease in the specific binding to the fractionated components, compared to the binding levels obtained after 2.5 hr of centrifugation. Furthermore, when solubilized material bound to [^{3}H]bremazocine, [^{3}H]EKC, or [^{3}H]diprenorphine was then subjected to sucrose density centrifugation, only very low levels of specific opiate binding could be detected. Bound radioactivity was measured in 0.5 ml of each fraction. These low levels of binding are probably due to a rapid dissociation of the bound complex under these conditions. Therefore, all binding studies were carried out after sucrose gradient centrifugation of unbound soluble material. Under optimal conditions, the recovery of [^{3}H]bremazocine binding from the gradient usually exceeded 100% and even reached levels of 150%–200% of the binding to the unfractionated soluble material. This increased level of binding may be largely due to the lower concentrations of digito-

Table 1. Specific binding of ^{3}H-labeled opioids to guinea pig brain and cerebellar membranes

	Etorphine	EKC	Bremazocine	DAMGE	DADLE
			Brain		
Unblocked	186 ± 12	75 ± 4	210 ± 11	31 ± 3	42 ± 3
Blocked	92 ± 4	32 ± 5	101 ± 8		
			Cerebellum		
Unblocked	115 ± 10	37 ± 5	108 ± 3	1.5 ± 1	1.7 ± 1
Blocked	98 ± 6	33 ± 4	92 ± 2		

Specific binding of ^{3}H-labeled opioid ligands (2 nM) was determined in the absence (unblocked) and presence (blocked) of DAMGE and DADLE (100 nM each). The results represent the mean ± SEM of three experiments and are expressed as fmol bound per mg of protein.

nin (<0.02%) present after fractionation. In addition, we have found that sucrose (3%–10%; wt/vol) enhances, by 20%–40%, the specific binding of [^{3}H]opioids to the soluble material. The reason for this enhancement is not clear at the present time, but it may represent a stabilization of the soluble proteins.

After sucrose gradient centrifugation of soluble material from guinea pig brain, [^{3}H]bremazocine (3 nM) displayed two major peaks of binding at fractions 3–4 (peak A) and 7–9 (peak B). Essentially, a single peak of binding at the location of peak A was obtained when binding was carried out in the presence of 100 nM DAMGE and 100 nM DADLE. As shown in Fig. 1, the addition of μ and δ agonists resulted in a 90% decrease of the binding of [^{3}H]bremazocine at peak B, whereas binding at peak A was virtually unaffected. Binding of [^{3}H]bremazocine to 11 fractions of solubilized material from guinea pig cerebellum revealed only 1 major peak at fractions 3–4, which coincides with peak A obtained on fractionation of guinea pig brain extracts (Fig. 1). In a previous study (28), we have shown that binding of [^{3}H]DAMGE and [^{3}H]DADLE to fractionated soluble material from guinea pig brain revealed identical peaks at fractions 7–9 (peak B of bremazocine binding) but was not detectable at fractions 3–4 (peak A of bremazocine binding).

To ascertain the binding characteristics of peaks A and B, various agonists were tested for their ability to inhibit [^{3}H]bremazocine binding at each peak. Fifty percent inhibition of [^{3}H]bremazocine binding at both peaks was achieved by low concentrations of bremazocine (IC_{50} = 2.0 and 1.3 nM at peaks A and B, respectively) and EKC (IC_{50} = 10 and 15 nM at peaks A and B, respectively). However, the selective μ ligand, DAMGE, and the relatively selective δ ligand, DSLTE, were unable to displace >50% of [^{3}H]bremazocine binding at peak A, even at a concentration of 10 μM. On the other hand, a 50% inhibition of [^{3}H]bremazocine binding at peak B was achieved by either 20 nM DAMGE or 8 nM DSLTE (Fig. 2; Table 2).

A comparison of the affinities of the various opioid ligands to soluble vs. membrane-bound receptors was made by turning to guinea pig cerebellum as a source of membrane-bound κ receptors and to rat brain preparation for membrane-bound μ and δ receptors. Opioid binding sites in rat brain correspond mostly to μ and δ sites (33). Fifty percent inhibition of [^{3}H]bremazocine binding to both preparations (guinea pig cerebellum and rat brain) was achieved by low concentrations of EKC (IC_{50} values of 7.0 and 6.3 nM) and bremazocine (IC_{50} values of 1.0 and 1.7 nM). In contrast, DAMGE and DSLTE displayed extremely low affinity (IC_{50}, >10 μM) for [^{3}H]bremazocine binding sites in guinea pig cerebellum, but were able to displace 50% of [^{3}H]bremazocine binding to rat brain membranes at a concentration range of 25–30 nM. As indicated in Table 2, the affinities of the various opioid ligands for κ vs. μ/δ membrane-bound receptors correspond rather well to the affinities of these ligands for the two soluble binding components (peaks A and B) from guinea pig brain.

Further similarities between the nature of the soluble and membrane-bound receptors were found by measuring inactivation of specific opioid binding after treatment with heat, trypsin, and *N*-ethylmaleimide. Pretreatment of either peak

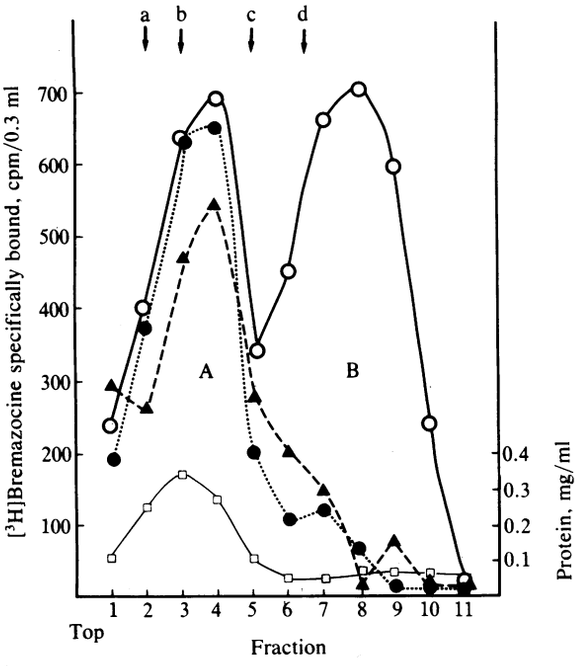

FIG. 1. Bremazocine binding to fractions of solubilized guinea pig brain and cerebellar membranes after sucrose gradient centrifugation. Fractions from brain were assayed for specific [^{3}H]bremazocine (3 nM) binding in the absence (○) and presence (●) of DAMGE and DADLE (100 nM each). Fractions from cerebellum were assayed under identical conditions, in the absence of DAMGE and DADLE (▲). The protein concentration (□) was assayed for each fraction. Protein markers for the determination of $s_{20,w}$ were as follows: a, catalase; b, thyroglobulin (monomer); c, ferritin; d, thyroglobulin (dimer). Each point for [^{3}H]bremazocine binding is the mean of at least three assays, which differed from each other by <15%.

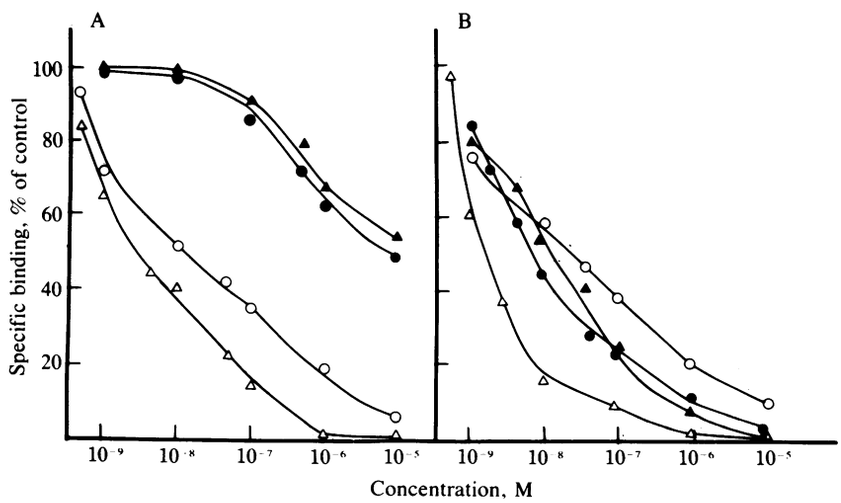

FIG. 2. Displacement of [^{3}H]bremazocine binding to soluble opioid binding sites from guinea pig brain by various opioid ligands. After sucrose density gradient centrifugation, fractions 3–4 (peak A) and fractions 7–9 (peak B) were pooled separately. The ability of opioid ligands to compete for [^{3}H]bremazocine (2.5 nM) binding was determined using 6–8 concentrations of unlabeled ligand. Control specific binding was 1100 ± 120 cpm/0.4 ml from either peak. Each displacement curve represents the mean of three assays, each carried out in triplicate. △, Bremazocine; ○, EKC; ▲, DAMGE; ●, DSLTE. (*A*) Experiments carried out on peak A. (*B*) Experiments carried out on peak B.

Table 2. Potencies of opioid ligands in the displacement of [^{3}H]bremazocine specific binding to sucrose gradient-fractionated soluble binding sites and membrane-bound opioid binding sites

Ligand	Soluble fraction from guinea pig brain		Membrane	
	Peak A	Peak B	Guinea pig cerebellum	Rat brain
DAMGE	≈10,000	20 ± 2	>10,000	25 ± 3
DSLTE	≈10,000	8 ± 1	>10,000	30 ± 4
EKC	10 ± 2	15 ± 2	7 ± 2	6.3 ± 0.5
Bremazocine	2.0 ± 0.3	1.3 ± 0.2	1 ± 0.1	1.7 ± 0.2

IC_{50} values (nM) for displacement of [^{3}H]bremazocine binding (2.5 mM) to the soluble opioid binding fractions (peak A and B) from guinea pig brain by various opioid ligands were determined from data represented in Fig. 2. IC_{50} values (nM) for displacement of [^{3}H]bremazocine binding (2.5 nM) to guinea pig cerebellum or rat brain membranes were determined from competition studies using 5–7 concentrations (0.5–10,000 nM) of unlabeled ligand. All binding assays were carried out under identical conditions. Data represent the mean ± SEM of at least three determinations.

A or B at 60°C for 20 min decreased the binding of [^{3}H]bremazocine by 84%. Treatment of the soluble binding components with trypsin (20 μg/ml; 30°C; 20 min) or *N*-ethylmaleimide (0.5 mM; 30°C; 20 min) resulted in 75% and 65% inhibition, respectively, of [^{3}H]bremazocine binding at peaks A and B. Almost identical results were obtained for the binding of [^{3}H]bremazocine to guinea pig cerebellum and rat brain membranes when these were exposed to the same treatments.

To estimate the apparent molecular weight of digitonin-solubilized binding components in peaks A and B of the sucrose gradient fractionation, we calculated their sedimentation coefficients ($s_{20,w}$) and those of known protein markers (34). The $s_{20,w}$ of peak A is 19 *s* and that of peak B is 34*s*–39*s*. By plotting the calculated *s* values of the protein markers against their known molecular weights, we estimated the apparent molecular weight of digitonin-solubilized κ binding component (peak A) to be ≈400,000 and that of μ/δ binding component (peak B) to be 750,000–875,000.

DISCUSSION

The present study provides evidence for the presence of the three major types of opioid binding sites, μ, δ, and κ, in digitonin-solubilized preparations from guinea pig brain. It also extends our preliminary finding (28), indicating the separation of two distinct soluble peaks of opioid binding activity after sucrose gradient fractionation. Evidence is provided for the separation of κ sites from a mixture of μ and δ sites. A separation of the latter has not yet been achieved.

The high affinity with which the highly selective μ ligand, DAMGE, binds to peak B leaves little doubt about the presence of μ binding sites. However, the evidence for the presence of δ sites requires special discussion, because highly selective δ ligands equivalent to DAMGE for μ sites were not yet available for this research. Moreover, the presence of δ sites in soluble extracts of brain membranes had not previously been reported. The presence of δ sites in peak B is strongly suggested by the following evidence: (*i*) DSLTE, a peptide with 7- to 10-fold higher affinity for δ than μ binding sites (35), binds to peak B with an IC_{50} value of 8 nM ($K_i = 4 \times 10^{-9}$ M, assuming K_d of bremazocine of 1×10^{-9} M), which is very close to the published K_d for DSLTE for δ sites and about 1/8th the K_d for μ sites (35); (*ii*) it is generally accepted that rat brain membranes contain a mixture of μ and δ sites (in a ratio of ≈60:40) with very few (10%–12%) κ sites. The IC_{50} of DSLTE for the displacement of [^{3}H]bremazocine binding from rat membranes was 20 nM. This decrease of IC_{50} in peak B compared to rat membranes suggests that peak B contains a proportion of δ sites at least as high as that present in rat brain membranes; (*iii*) in two recent experiments (unpublished results) we have been able to show that the binding of [^{3}H]bremazocine to sites in peak B remaining after addition of 20 nM DAMGE is readily displaceable by DSLTE with IC_{50} between 2 and 4 nM, somewhat lower than in the absence of DAMGE. This again suggests that a large portion of the sites not blocked by this rather high concentration of DAMGE represents δ sites. We conclude from this evidence that peak B contains a mixture of μ and δ sites.

The following evidence supports the achievement of the physical separation of solubilized opioid binding sites into κ sites in one peak and into μ and δ sites in the other. First, binding of [^{3}H]bremazocine, a "universal" ligand that displays a similar affinity for μ, δ, and κ receptors (10), to the fractionated soluble components from guinea pig brain revealed two peaks of specific binding. Binding at peak A was not affected by what should have been saturating concentrations of μ and δ ligands. However, the addition of saturating concentrations of μ and δ ligands to peak B resulted in 80%–90% inhibition of [^{3}H]bremazocine binding. Second, as indicated in this study and in agreement with previous findings (32), the guinea pig cerebellum contains essentially a single type of opioid binding site—namely, κ. Binding of [^{3}H]bremazocine to gradient-fractionated solubilized cerebellum revealed a single peak of binding. This peak was superimposable with peak A of [^{3}H]bremazocine binding from fractionated solubilized guinea pig brain. These findings strongly suggest that peak A corresponds to κ binding sites. Third, the high affinity of DAMGE (IC_{50}, 20 nM) and DSLTE (IC_{50}, 8 nM) to [^{3}H]bremazocine binding sites at peak B (as already discussed) together with their inability to displace >50% of the binding at peak A, even at a concentration of 10 μM, strongly suggests that peak B contains both μ and δ sites and further supports the presence of a vast preponderance of κ sites in peak A.

The affinities of bremazocine, EKC, DAMGE, and DSLTE for the soluble κ, μ, and δ binding components are similar to their affinities for the corresponding membrane-bound receptors present in guinea pig cerebellum (κ) and rat brain (mostly μ and δ sites). These results imply that the soluble opioid receptors retain high affinity and selectivity for various opioids similar to that seen in membrane-bound receptors. Additional similarities between the properties of the soluble and membrane-bound receptors were shown by treatment with heat, trypsin, and *N*-ethylmaleimide. Inactivation of specific [^{3}H]bremazocine binding to soluble components occurred to an extent similar to that observed with membrane-bound receptors.

Most of the extracted proteins appear in peak A, yielding little purification for the κ sites present in this peak. Peak B, however, exhibits a specific binding activity (890 ± 45 fmol per mg of protein) 10-fold higher than peak A, representing some purification for the μ and δ mixture present in this peak by the single step of density-gradient centrifugation.

The apparent sedimentation coefficient obtained for the κ binding component is 19 *s* and that for the mixture of μ and δ binding components is 34*s*–39*s*. Accordingly, the approximate molecular weights of these binding components are 400,000 and 750,000–875,000, respectively. The apparent sedimentation coefficient of a digitonin–protein complex is likely an overestimation of the actual $s_{20,w}$ value of the protein alone, because digitonin micelles have been estimated to be 6.3 *s* (36). In fact, the calculated $s_{20,w}$ values for marker proteins, prepared in 0.5% digitonin solution, are 15%–30% higher than their reported values. The amount of digitonin bound to the protein cannot be determined, because the partial specific volume of digitonin, 0.738 ml/g (36), is similar to that of protein, and therefore the partial specific volume of

the detergent–protein complex does not change with increasing amount of detergent bound.

In previous studies, we generally found a molecular weight of 300,000–400,000 for digitonin-solubilized opioid binding sites as determined by gel filtration (20, 21). The apparent molecular weight reported here for the κ sites is in excellent agreement with these earlier results. However, the molecular weight of peak B is about twice as great. The reason for this is not understood, but it could be the result of polymerization of these sites when the digitonin concentration is very low. It is also possible that the large species in peak B represent complexes of opioid binding sites with other components of the receptor system, such as coupling proteins and enzymes. Earlier molecular weight determinations (20, 21) were carried out with unfractionated opioid binding sites on gel columns and could thus represent an average molecular weight slightly lower than the average of the two peaks on the sucrose gradient, a difference that could easily be due to the different methods used. Molecular weights of 600,000–800,000 have also been reported for soluble opioid receptors by others (37). The most important result reported here is the physical difference between binding sites that has permitted their separation on a sucrose density gradient. This finding supports the hypothesis that κ sites represent molecular species different from those of μ and δ sites. It should be pointed out that different molecular species does not necessarily imply a different primary gene product (polypeptide), but it could also represent differences in the degree of polymerization of the same polypeptide subunit or in post-translational modifications, such as glycosylation, lipidation, or phosphorylation.

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