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Solubilization and Preliminary Characterization of *Mu* and *Kappa* Opiate Receptor Subtypes from Rat Brain

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SUMMARY

The opiate agonists [^{3}H]dihydromorphine (DHM, μ-selective ligand), [^{3}H]bremazocine (potent κ ligand), and [^{3}H]etorphine bound stereospecifically, with high affinity, and reversibly to partially purified 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate (CHAPS)-solubilized extract from rat brain membranes. Recoveries of the three binding activities were as follows: [^{3}H]DHM, 47%; [^{3}H]bremazocine, 55%; and [^{3}H]etorphine, 17%. Each ligand exhibited (by Scatchard analysis) binding to a class of high-affinity sites (K_d = 0.8–2 nM). Hill analyses revealed Hill coefficients of n = 1.1–1.3. Many of the properties of solubilized brain opiate receptors are similar to those of membrane-associated opiate receptors. Opiate binding in soluble fractions was inhibited by a variety of protein-modifying agents, including trypsin, proteinase K, and *N*-ethylmaleimide as well as by heat treatment (60°, 15 min). The relative potencies of a series of opiate narcotic agonists and antagonists in displacing 2 nM [^{3}H]etorphine binding to the CHAPS-solubilized extract was similar to that determined for rat brain homogenates. In contrast, D-Ala^2,D-Leu^5-enkephalin (DADLE, putative δ-selective ligand) exhibited a much lower affinity for solubilized brain opiate receptors than for the membrane-bound receptors unless assayed in the presence of manganese chloride, sodium chloride, and GTP. *Mu* agonist binding to solubilized receptors was inhibited relatively selectively by sodium and guanyl nucleotides. These findings lend support to the pharmacological relevance of the solubilized opiate-binding component(s). The pI of the solubilized brain opiate receptor(s) was estimated by liquid isoelectrofucusing to be pH 4. The sizes of the solubilized, prelabeled [^{3}H]etorphine-receptor complex of the solubilized μ and κ receptor subtypes, as assayed by stereospecific binding of [^{3}H]DHM and [^{3}H]bremazocine binding, respectively, were estimated by molecular exclusion chromatography. The [^{3}H]etorphine-receptor complex migrated as a broad radioactive peak at a position corresponding to a protein of Stoke radius 63 Å. A secondary peak of radioactivity was observed at the salt peak. *Mu* receptor activity chromatographed as two major peaks. The first of these eluted just behind, but significantly separated from, the protein void peak and corresponded to a Stokes radius of 70 Å; the second eluted just ahead of the salt peak and corresponded to a radius of less than 20 Å. *Kappa* receptor activity eluted at positions corresponding to macromolecules of 50 Å and ≤20 Å. Together, these findings indicate that selective μ and κ ligands interact with high molecular weight species of somewhat different sizes as well as a lower molecular weight species, which may represent a common subunit that can bind both ligands.

INTRODUCTION

Increasing biochemical evidence indicates that the wide spectrum of opiate pharmacological actions is mediated by interaction with heterogeneous receptor subtypes (for reviews see refs. 1 and 2). The μ receptor has been identified as the high-affinity site at which morphine-like opiates produce analgesia and a variety of other classical opiate effects (3, 4). The δ receptor was defined as a site that is relatively selective for the naturally occurring enkephalins (5). Recent biochemical studies provide evidence for two additional opiate receptor subtypes which were originally proposed on the basis of neurobehavioral studies. Ketocyclazocine-like opiates apparently produce analgesia as well as their unique ataxic and sedative effects through an interaction with the κ receptor. SKF-10,047 (*N*-allylnorcyclazocine) and related opiates produce psychotomimetic and dysphoric effects by an interaction withthe σ receptor (3, 4). Many opiate drugs interact at multiple receptor sites. Thus, the

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complex neuropharmacological actions of a particular opioid ligand would appear to reflect its various potencies at the μ, δ, κ, and σ receptor subtypes.

Kosterlitz and his co-workers (5) first demonstrated pharmacological and biochemical evidence for the existence of heterogeneous opiate receptor populations. More recently, further studies involving competition of ligands for radiolabeled opiate binding sites in brain (6–10) and cross-protection studies involving inactivation of opiate binding by phenoxybenzamine (11) or selective sulfhydryl reagents (12) have provided considerable biochemical evidence for the μ and δ subtypes and indicate that these have somewhat different distributions throughout the central and peripheral nervous systems. The distribution of μ and δ receptors has been confirmed in the case of the central nervous system by light microscopy autoradiography (13), and in the case of the peripheral nervous system by the twitch assay on isolated tissue strips (5, 11). Attempts to establish the presence of κ and σ receptors using radiolabeled opiates have been more difficult, primarily because of the lack of highly specific ligands for these receptor subtypes. Both cross-protection studies (14, 15) and direct binding studies using ^{3}H-labeled κ and σ drugs in the presence of selective μ and δ blockers (16, 17) have provided support for the concept of distinct κ and σ binding sites. Snyder and his co-workers (18) have used [^{3}H]bremazocine and [^{3}H]ethylketocyclazocine in the presence of morphine and DADLE[1] to map κ receptors in brain; the distribution of these was shown to differ from that of μ or δ receptors in layers V and VI of the cerebral cortex. Pert and co-workers (19) have used [^{3}H]phencyclidine (putative σ ligand) in an autoradiography study to map σ receptors in the brain. [^{3}H]Phencyclidine binding sites, visualized by tritium-sensitive LKB film and analyzed by computerized densitometry, exhibit a unique distribution quite unlike that of the μ, δ, or κ receptors. Distinct high-affinity benzomorphan binding sites, which do not bind enkephalin or morphine, have been demonstrated to be present in the NCB-20 neuroblastoma-brain hybrid cell line (20); these have been identified with the putative κ and σ receptors.

The question arises as to the molecular basis of opiate receptor heterogeneity. The four receptor subtypes may be distinct polypeptide entities or they may represent a single protein receptor in differing conformational or aggregational states. Solubilization and characterization of brain opiate receptors provides a direct means to elucidate the molecular properties that distinguish the subtypes. Solubilization of active opiate receptors from tissues bearing a single receptor subtype have been reported. Opiate receptors have been solubilized in the antagonist binding state from toad brain (21) (source of κ receptors) and more recently from mammalian brain (22), using digitonin; and δ receptors, from the neuronal cell line NG-108-15, using the novel zwitterionic detergent CHAPS (23). In the present study, we have used CHAPS to solubilize opiate receptors from rat brain, a source of heterogeneous receptor subtypes, in relatively high yields. The use of a relatively selective μ ligand and a κ ligand in the presence of μ and δ blockers has enabled a preliminary characterization of the molecular properties of those receptor subclasses.

[1] The abbreviations used are: DADLE, D-Ala^2,D-Leu^5-enkephalin; CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate; PEG, polyethyleneglycol; NEM, *N*-ethylmaleimide; DHM, dihydromorphine.

EXPERIMENTAL PROCEDURES

Materials. [^{3}H]Etorphine (18 Ci/mmole) was generously provided by the National Institute of Drug Abuse (Rockville, Md.). The [^{3}H]etorphine was determined to be greater than 95% pure by analysis on thin-layer chromatography using the solvent system ethyl acetate/hexane/ethanol/ammonia (60:25:14:1). The concentration of the radiolabeled drug was determined independently by ultraviolet absorption as previously described (24). [^{3}H]Dihydromorphine (76 Ci/mmole) was obtained from New England Nuclear Corporation (Boston, Mass.). [^{3}H]Bremazocine (24 Ci/mmole) and bremazocine were generous gifts of Dr. D. Roemer, of Sandoz, Ltd. (Basel, Switzerland).

Levorphanol and dextrorphan were gifts from Hoffmann-La Roche (Nutley, N. J.); cyclazocine and pentazocine were gifts from Sterling Winthrop Research Laboratories (New York, N. Y.). Etorphine and normorphine were provided by the National Institute of Drug Abuse. Naloxone was obtained from Endo Laboratories (Garden City, N. Y.). Trypsin, soybean trypsin inhibitor, proteinase K, PEG 6000, NEM, and dithiothreitol were from Sigma Chemical Company (St. Louis, Mo.). CHAPS was obtained from Calbiochem (San Diego, Calif.). DADLE and all other peptides were purchased from Peninsula Laboratories. DE 81 (DEAE-cellulose) discs were from Whatman Inc. (Clinton, N. J.).

Solubilization. Solubilization of brain opiate receptors was carried out by a modification of the method of Simonds *et al.* (23). P_2 (mitochondrial/synaptosomal) membranes were prepared from whole brains (minus cerebellum) of male Sprague-Dawley rats (150–200 g) as described previously (24). The P_2 pellet was resuspended in 2 volumes of cold 10 mM Tris-HCl buffer (pH 7.5). CHAPS was added to a final concentration of 10 mM, and the suspension was homogenized (10 strokes) with a ground-glass tissue grinder and centrifuged at 105,000 × *g* for 60 min at 4°. The resulting clear supernatant fluid was carefully removed from a solid pellet and the cloudy suspension which floated just above the pellet. Opiate receptors in the CHAPS-soluble fraction were partially purified by a PEG precipitation method (25). Briefly stated, the CHAPS extract was adjusted to pH 5.6 by addition of 1 M KAc buffer (pH 5.6); PEG was then added to a final concentration of 17%. The receptor preparation was incubated at 4° for 15 min, centrifuged at 25,000 × *g* for 20 min, and washed once with 1 volume of 10 mM Tris-HCl 1 mM CHAPS/1 mM dithiothreitol. The pellet, which contained opiate binding activity, was then resuspended in 0.3 times the original volume with 10 mM Tris-HCl (pH 7.4) by sonication for 20 sec. For binding assays, the receptor sample was adjusted to a final detergent concentration of 1 mM (see below).

The PEG procedure resulted in an increase in specific opiate binding from 30% to approximately 55–80% of total binding. Except where specifically stated, the binding experiments described below were carried out with solubilized, partially purified opiate receptors from rat brain.

Molecular exclusion chromatography. The size of the [^{3}H]opiate receptor complexes and of μ and κ binding activity were estimated by molecular exclusion chromatography. P_2 membranes were prepared from rat brain, solubilized with CHAPS, and centrifuged at 100,000 × *g* as described above. A 3-ml sample of the supernatant (labeled or unlabeled) was applied to a Sepharose 6B CL (Pharmacia) column (1 × 85 cm) and eluted with 0.32 M sucrose/1 mM CHAPS in 10 mM Tris-HCl at a flow rate of 10 ml/hr at 4°. Two-milliliter fractions were collected. In the case of prelabeled CHAPS extract, the P_2 membranes were suspended in 4 volumes of 10 mM Tris-HCl buffer (pH 7.5) (w/v) and incubated with 2 nM [^{3}H]etorphine or [^{3}H]bremazocine for 30 min at 37°, washed, solubilized with 10 mM CHAPS, and applied to the column. After elution, aliquots (0.5 ml) from every second fraction were counted by liquid scintillation spectrometry, and the absorbance was

measured at 280 nm. For direct binding experiments, the CHAPS supernatant was applied directly to the column, and fractions were assayed for absorbance at 280 nm and for [^{3}H]DHM or [^{3}H]bremazocine binding as described below.

Isoelectric focusing. CHAPS-solubilized receptor preparations were fractionated by liquid isoelectric focusing with a 110-ml electrofocusing column at 4°. A stabilizing gradient of 0–50% sucrose containing 1 mM CHAPS was used with carrier ampholytes (LKB) at a concentration of 1% in the pH range 3–10. Because of inactivation of binding activity upon migration to an acidic pH, isoelectric focusing was carried out with CHAPS extract that had been prelabeled with [^{3}H]etorphine. The P_2 pellet was resuspended in 4 volumes of 10 mM Tris (pH 7.4) (5 mg of protein per milliliter) and was incubated with [^{3}H]etorphine (2 nM) for 1 hr at 4°. The membranes were then washed twice, resuspended to a final protein concentration of 15 mg/ml, and treated with CHAPS as above. After centrifugation at 100,000 × *g*, the supernatant was introduced midway into the column as the sucrose gradient was being made; electrofocusing was performed at 1000 V for 40 hr. Two-milliliter fractions were collected at a flow rate of 2 ml/hr.

Opiate binding. Binding assays of intact membranes were carried out as described (26). Assays of the solubilized receptors were performed with either the PEG or the DEAE method. In the PEG precipitation method, tubes in triplicate containing 2 nM tritiated ligand, solubilized receptor, 1 mM CHAPS (final concentration) and 10 mM Tris-HCl (pH 7.4) in the presence of dextrorphan (1.0 μM) or levorphanol (1.0 μM) (0.5 ml final volume) were incubated at 37° for 20 min. At the end of the incubation period, 40 μl of 1% γ-globulin was added to each tube, followed by 1 ml of 23% PEG 5000 in 100 mM KAc (pH 5.6). Samples were incubated for an additional 10 min at 4°. Free ligand was separated from protein-bound ligand by filtration under reduced pressure through Whatman GF/B filters. The filters were washed rapidly with two aliquots of 5 ml of 7% PEG/10 mM KAc (pH 5.6). Specific μ receptor binding was assayed as above using [^{3}H] dihydromorphine. In the case of κ receptor assays, [^{3}H]bremazocine was incubated with solubilized receptor in the presence of 40 nM normorphine and 100 nM DADLE (in order to block μ and δ receptor binding and to direct [^{3}H]bremazocine to κ receptors). Specific κ binding is defined as this binding minus [^{3}H]bremazocine binding in the presence of these nonlabeled ligands and also 10 μM cyclazocine. We have chosen cyclazocine as displacer, as we have previously shown this drug to bind potently to κ and σ receptors (2, 16). In the case of δ receptor assays, the conditions were the same as for μ assays, except that 3 mM $MnCl_2$, 10 μM GTP, and 100 mM NaCl were included in all samples.

For the DEAE method, the incubation mixture was as described for the PEG assay with the exception that the final volume was 120 μl; a 100 μl-sample was applied to each dry DE 81 (DEAE cellulose) disc. Discs were washed extensively at 4° with 10 mM Tris-HCl (pH 7.4) for 30 min. In all cases, nonspecific binding was defined as the difference between binding of the radioactive ligand alone and that in the presence of 1 μM nonradioactive ligand as indicated. The DEAE method is based on the difference in charge properties of the acidic receptor protein and the basic tertiary ammonium group of many opiate alkaloids. Thus, at pH 7.4, the receptor is expected to bind the anion exchange discs, whereas the ligand does not. Protein concentration was measured either by absorbance at 280 nm or by the method of Lowry *et al.* (27) with bovine serum albumin as the standard.

For studies with protein-modifying reagents, aliquots of PEG-purified CHAPS extract (0.5 ml, 150 μg of protein) were incubated for 15 min in 10 mM Tris-HCl (pH 7.4, 30°) in the presence of trypsin (10 μg/ml), proteinase K (10 μg/ml), or NEM (0.5 mM), or with no added reagent. In the case of NEM, the reaction was stopped by the addition of excess dithiothreitol (2.5 mM) (26). Samples were then incubated for an additional 20 min at 37° with [^{3}H]etorphine (2 nM) in the absence or presence of levorphanol (1.0 μM) and filtered as above.

RESULTS

Equilibrium binding of radiolabeled opiates to CHAPS-soluble extracts from rat brain. Stereospecific [^{3}H]DHM (putative μ ligand) binding, defined as binding in the presence of 10 μM dextrorphan minus binding in the presence of 10 μM levorphanol, was saturable with respect to radiolabeled ligand concentration (Fig. 1). Specific binding constituted approximately 75% of total binding (at 1.0 nM ^{3}H-labeled ligand) and 54% of total binding (at 20 nM ^{3}H-labeled ligand). Half-maximal binding occurred at approximately 5 nM [^{3}H]DHM. In contrast, nonspecific binding, indicated by the binding of [^{3}H]DHM in the presence of 10 μM levorphanol, was not saturable and increased linearly with increasing ^{3}H-labeled ligand. Scatchard analysis of these data (Fig. 1, *inset*) revealed a linear plot consistent with binding to a single class of high-affinity sites (37 ± 3 fmoles/mg of protein) with an apparent affinity of 2 ± 0.5 nM (r = 0.96). A comparison of this receptor density with that obtained in the case of [^{3}H]DHM binding to rat brain homogenate high-affinity sites [B_{max} = 78 fmoles/mg protein (28)] indicates a yield of 47% for the solubilization step.

Binding of [^{3}H]bremazocine, a potent κ agonist (29) to CHAPS-soluble extract from rat brain was also examined. In Fig. 2A, specific binding is defined as the difference between [^{3}H]bremazocine bound in the absence and presence of 10 μM cyclazocine. Saturation analysis again revealed binding to a class of high-affinity sites; half-maximal binding was achieved at approximately 0.8 nM. Scatchard analysis (Fig. 2A, *inset*) indicated binding to a class of sites (76 ± 5 fmoles/mg of protein) with an affinity of 0.8 ± 0.05 nM (r = 0.98). In Fig. 2B, specific binding is the difference between [^{3}H]bremazocine binding in the presence of 40 nM normorphine, with 100 nM DADLE and [^{3}H]bremazocine binding in the presence of normorphine, with DADLE, and 10 μM cyclazocine. These conditions have been shown to block binding to μ and δ receptors and to direct [^{3}H]bremazocine binding to

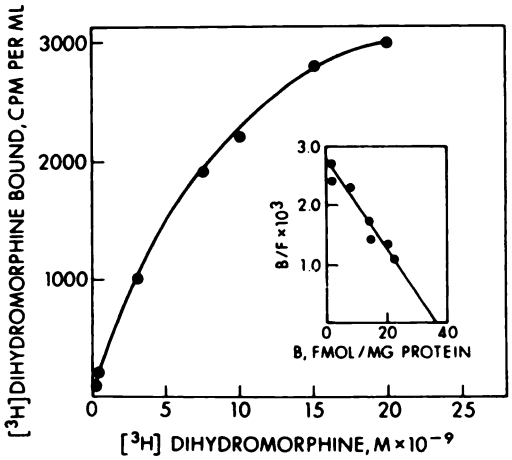

FIG. 1. *Equilibrium binding of [^{3}H]dihydromorphine to solubilized opiate receptors as a function of ligand concentration and (inset) as a Scatchard analysis*

Aliquots of PEG-purified CHAPS extract (0.5 ml, 150 μg of protein) were incubated in triplicate at 37° for 20 min with various concentrations of radiolabeled opiate in the presence of dextrorphan (10 μM) or levorphanol (10 μM). Samples were precipitated and filtered as described in the text. Specific binding, defined as binding in the presence of dextrorphan minus binding in the presence of levorphanol, is reported. This experiment was replicated three times. The Scatchard plot was fit by a straight line using linear regression analysis (r = 0.96).

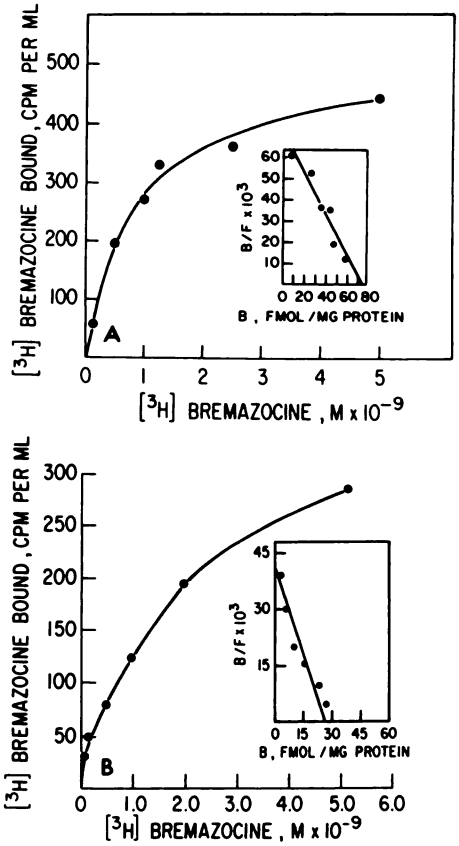

FIG. 2. *Equilibrium binding of [^{3}H]bremazocine to solubilized opiate receptors (A) unblocked or (B) after suppression of μ and δ receptor binding as a function of ligand concentration and (insets) as Scatchard analyses*

Aliquots of PEG-purified CHAPS extract (0.5 ml, 150 μg of protein) were incubated at 37° for 20 min with various concentrations of [^{3}H] bremazocine (in A) in the absence or presence of cyclazocine (10 μM). Samples were precipitated and filtered as described in the text. Specific binding, defined as total binding minus binding in the presence of cyclazocine, is reported. In B, conditions were the same as in A, except that 40 nM normorphine and 100 nM DADLE were included in all samples. In that case, specific binding was defined as [^{3}H]bremazocine binding in the presence of normorphine and DADLE minus binding in the presence of normorphine, DADLE, and cyclazocine. Each experiment was replicated three times. The Scatchard plot was fit by straight lines using linear regression analysis (in A, $r = 0.98$; in B, $r = 0.90$).

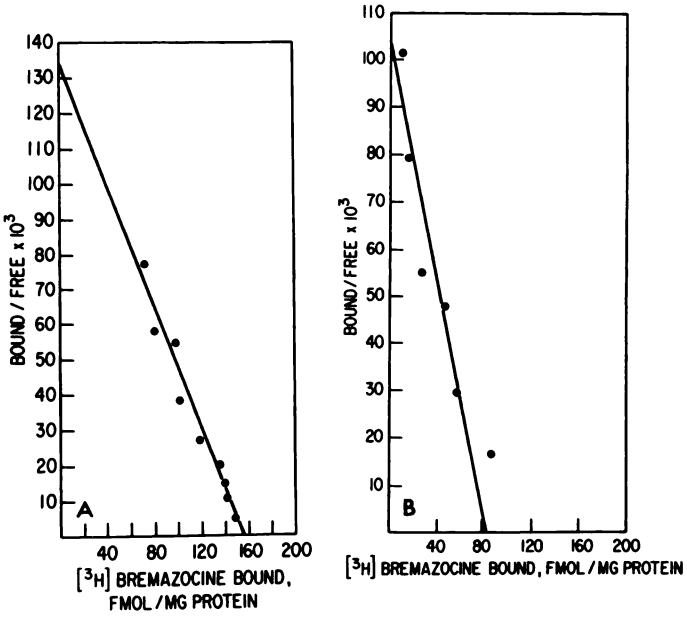

FIG. 3. *Equilibrium binding of [^{3}H]bremazocine to rat brain membranes (A) unblocked or (B) after suppression of μ and δ receptor binding*

Aliquots of whole rat brain homogenate (0.5 ml, 350 μg of protein) were incubated at 37° for 20 min with various concentrations of [^{3}H] bremazocine (in A) in the absence or presence of cyclazocine (10 μM). Samples were filtered as described in the text. Specific binding, defined as total binding minus binding in the presence of cyclazocine, is reported. In B, conditions were the same as in A except that 40 nM normorphine and 100 nM DADLE were included in all samples. In that case, specific binding was defined as [^{3}H]bremazocine binding in the presence of normorphine and DADLE minus that in the presence of normorphine, DADLE, and cyclazocine. Each experiment was replicated three times; data were fit by straight lines using linear regression analyses (in A, $r = 0.95$; in B, $r = 0.94$).

κ receptors (17, 18, 30). K_D and B_{max} under these conditions are 0.7 ± 0.1 nM and 25 ± 5 fmoles/mg of protein ($n = 3$, $r = 0.90$). Omission of DADLE from the binding samples resulted in data essentially identical with those shown in Fig. 2B. In order to compare these results with the case of the membrane-bound receptors, binding of [^{3}H] bremazocine (unblocked) to rat brain membranes (Fig. 3A) was also examined. Scatchard analysis of the data afforded a K_D of 1.1 ± 0.3 nM B_{max} of 137 ± 11 fmoles/mg of protein ($n = 3$; $r = 0.95$). Binding of [^{3}H] bremazocine to membranes in the presence of normorphine (40 nM) and DADLE (100 nM) (Fig. 3B) occurred with an affinity of 0.78 ± 0.05 nM to 81 ± 9 fmoles of receptor per milligram of protein ($n = 3$; $r = 0.94$). Thus the yields for total solubilized [^{3}H]bremazocine binding proteins and for solubilized κ receptors were 55% and 31%, respectively. The ratio of solubilized μ and κ receptors was approximately 1.5:1, in agreement with that found for rat brain membranes (17).

Binding of [^{3}H]etorphine, a potent narcotic agonist which most probably interacts with similar potencies to μ, δ, κ, and σ opiate receptor subtypes (2), was also investigated. Saturation analysis (Fig. 4) revealed high-affinity, saturable binding; half-maximal binding was achieved at approximately 0.5 nM. When these data were analyzed as a Scatchard plot (Fig. 4, *inset*), binding to an apparent single class of sites (70 fmoles/mg of protein; $K_D = 1$ nM ($r = 0.90$) was observed. Comparison of this receptor density with that obtained in the case of rat brain homogenates [410 fmoles/mg of protein (28)] revealed a yield of 17% for the solubilization of [^{3}H]etorphine binding sites. The relatively low yield in this case may indicate that one or more of the solubilized [^{3}H] etorphine binding sites is not active under these assay conditions or is possibly inactivated during the solubilization step.

Very low specific binding of [^{3}H]DADLE, a putative δ ligand, to CHAPS-solubilized receptors was detected when assays were performed in 10 mM Tris-HCl buffer (pH 7.4). In contrast, when assays were performed in the presence of 3 mM $MnCl_2$, 10 μM GTP, and 100 mM NaCl (Fig. 5), high-affinity binding of [^{3}H]DADLE [$K_D = 0.6$ ± 0.2 nM; $B_{max} = 77 \pm 10$ fmoles/mg of protein ($n = 3$; $r = 0.91$)] was observed. These findings suggest that the δ

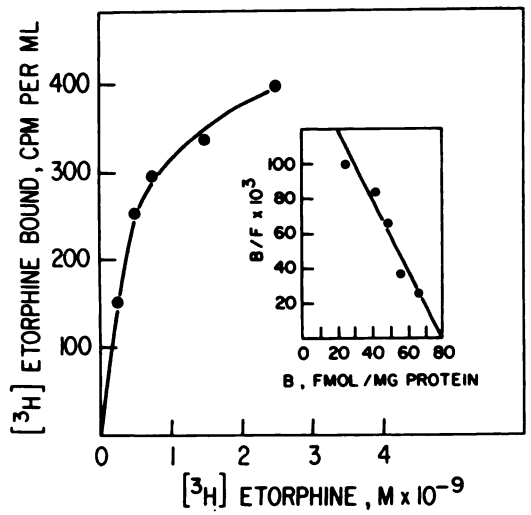

FIG. 4. *Equilibrium binding of [³H]etorphine to solubilized opiate receptors as a function of ligand concentration and (inset) as a Scatchard analysis*

Binding conditions were as described in Fig. 1. This experiment was replicated three times. The Scatchard plot was fit by a straight line using linear regression analysis ($r = 0.90$).

receptor is solubilized in an inactive configuration, and that it can be converted to its active conformation by these effector molecules. Bowen *et al.* (31) have suggested that these ionic conditions convert Type I opiate receptors of the striatum from the μ to the δ configuration.

Specificity of opiate binding to purified CHAPS extract from rat brain. Specific binding of [³H]etorphine to purified CHAPS extract varied linearly as a function of protein concentration between 0.04 and 0.4 mg of protein per milliliter (data not shown). Binding studies were routinely performed within this linear range. When the

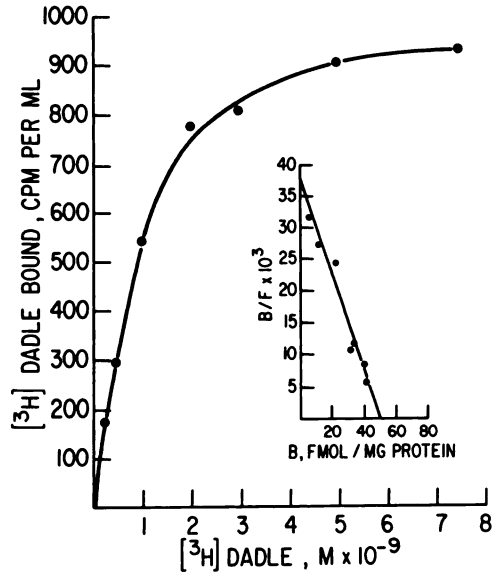

FIG. 5. *Equilibrium binding of [³H]DADLE to solubilized opiate receptors as a function of ligand concentration and (inset) as a Scatchard analysis*

Binding conditions were as described in Fig. 1, except that 3 mM $MnCl_2$, 10 μM GTP, and 100 mM NaCl were included in all samples. This experiment was replicated three times. The Scatchard plot was fit by a straight line using linear regression analysis ($r = 0.96$).

TABLE 1

Effect of various reagents on specific binding of [³H]etorphine to solubilized brain receptors

Specific binding of [³H]etorphine (2 nM) to solubilized receptors (0.5-ml samples, 0.3 mg of protein/ml) from rat brain was measured as described under Experimental Procedures. Data are the means ± standard error of the mean of three independent experiments.

Reagent	Concentration	Specific [³H]etorphine binding	
		fmoles/mg protein	% of control
Control	—	40 ± 2.3	100
Heat	60°, 15 min	1.2 ± 0.5	3
Trypsin	10 μg/ml	2.8 ± 1.0	7
Proteinase K	10 μg/ml	6.0 ± 1.5	15
NEM	0.5 mM	5.6 ± 1.0	14
GTP	10 μM	7.2 ± 0.2	18
	20 μM	3.2 ± 0.2	8
	50 μM	0.4 ± 0.1	1
ATP	20 μM	37 ± 2	92
NaCl	20 mM	37 ± 1	92
	50 mM	42 ± 3	105
LiCl	50 mM	41 ± 2	101
MgCl	20 mM	32 ± 2	79

data shown in Figs. 1, 2A, and 4 were replotted as Hill plots, Hill coefficients of 1.1, 1.2, and 1.3 were obtained for [³H]etorphine, [³H]DHM, and [³H]bremazocine, respectively. Incubation of the extract with trypsin (10 μg/ml) for 15 min at 30° decreased specific binding of [³H]etorphine (2 nM) by 93% relative to control samples incubated under the same conditions in the absence of trypsin (Table 1). Incubation (15 min, 30°) of proteinase K (10 μg/ml) also resulted in a significant inhibition (85%) relative to control binding. Pretreatment of the extract with the sulfhydryl reagent, NEM, (1 mM) for 15 min at 30°, followed by quenching with dithiothreitol (2.5 mM) reduced specific [³H]etorphine binding by 86%. Preincubation of the extract at 60° for 15 min decreased specific binding of [³H]etorphine (2 nM) by 97% relative to control samples incubated at 4°. Results essentially identical with those shown in Figs. 1, 2, 4, and 5 were obtained whether the separation of bound from free radiolabeled ligand was achieved using (a) PEG precipitation and filtration, (b) DE 81 discs, or (c) gel filtration on Sephadex G-25 columns (1 × 10 cm).

When the experiment described in Fig. 1 was carried out with P_2 membranes prepared from cerebellar tissue [a brain region with low opiate receptor density (2)], the binding of [³H]etorphine to solubilized receptors in the presence of dextrorphan (10 μM) was indistinguishable from that in the presence of levorphanol (10 μM).

Effects of opiates and opioid peptides on specific [³H] etorphine binding. The relative potencies of a series of opiates and opioid peptides in displacing [³H]etorphine binding from CHAPS extract is shown together with those for opiate receptor binding to brain membranes (Table 2). Of the opiates studied, bremazocine, a potent κ agonist (29), and etorphine, a very potent narcotic agonist in clinical and binding assays (32), were the most potent inhibitors of specific [³H]etorphine binding in soluble extracts of rat brain. The opioid peptide, DADLE, a putative δ ligand, and pentazocine, a weak narcotic analgesic, exhibited very weak affinities for this binding.

TABLE 2

Relative potencies of opiates in reducing [^{3}H]etorphine binding to brain opiate receptors

Whole rat brain minus cerebellum was used to prepare PEG-purified CHAPS extract, as described under Experimental Procedures. Aliquots of solubilized receptors (0.5 ml, 150 μg of protein) were incubated in triplicate at 37° for 20 min with 2 nM [^{3}H]etorphine (18,000 cpm) and with nonradioactive opioid compounds at eight concentrations in 10 mM Tris-HCl buffer (pH 7.4). One hundred percent binding is bound [^{3}H]etorphine in the presence of 10 μM dextrorphan minus bound [^{3}H]etorphine in the presence of 10 μM levorphanol and was 500 cpm per sample. Samples were precipitated, filtered, and assayed for radioactivity as described under Experimental Procedures. IC_{50} is defined as the concentration of drug which elicits half-maximal inhibition of specific [^{3}H]etorphine binding. Each determination represents the mean standard error of the mean of three independent experiments.

Whole rat brain minus cerebellum was used to prepare brain membranes as previously described (16). Aliquots of brain homogenates (1.0 ml, 0.75 mg of protein) were incubated in triplicate in 50 mM Tris-HCl (pH 7.4 at 37°) for 20 min with 2 nM [^{3}H]etorphine (36,000 cpm) and with nonradioactive opioid compounds at eight concentrations. Samples were filtered and assayed as described (16). One hundred percent binding and IC_{50} values are described above. Each determination represents the mean ± standard error of the mean of three independent experiments.

Drug	CHAPS-soluble Extract		Brain Membranes	
	IC_{50}	Relative potency[a]	IC_{50}	Relative potency[a]
	nM		*nM*	
Etorphine	1.0 ± 0.5	1	0.38 ± 0.05	1
Bremazocine	0.8 ± 0.2	1.2	0.35 ± 0.04	1.1
Levorphanol	1.2 ± 0.5	0.8	0.50 ± 0.02	0.8
D-Ala^2,N-*Me*-Phe^4-Met(*O*)-ol^5-enkephalin	2.6 ± 0.5	0.4	0.17 ± 0.02	2.2
Normorphine	10.1 ± 1.0	0.1	8.0 ± 0.5	0.05
DADL	1,200 ± 110	0.0008	7.8 ± 1.2	0.05
Pentazocine	1,300 ± 140	0.0008	69.0 ± 6	0.005
Dextrorphan	16,000 ± 1,500	0.0001	1,050 ± 100	0.0003

[a] Relative potencies are expressed relative to etorphine.

The IC_{50} of 1.2 μM observed for DADLE is in contrast to that found for displacement of [^{3}H]etorphine binding to rat brain membranes. This finding is consistent with our observation of low specific binding of [^{3}H]DADLE to CHAPS extract unless $MnCl_2$, GTP, and NaCl were present. Together, these findings suggest that δ receptors are present but are "masked" in the Tris-buffer extract. In contrast, D-Ala^2,*N-Me*-Phe^4,Met(*O*)ol^5-enkephalin, a relatively μ-selective peptide (9, 24), was rather potent in the inhibition of [^{3}H]etorphine binding under these conditions.

Soluble receptor-effector interactions. The modulation of opiate binding to CHAPS-solubilized receptors by guanyl nucleotides and salts was also examined (Table 1). As was observed in the case of brain membranes, the presence of GTP resulted in a significant inhibition of binding. In the absence of sodium, GTP (50 μM) reduced the binding of [^{3}H]etorphine by 99%. In contrast, this same concentration of GTP reduced binding of the narcotic antagonist [^{3}H]naloxone by less than 10% (data not shown). A variety of salts, including NaCl, LiCl, and $MgCl_2$, had only modest effects on [^{3}H]etorphine binding to solubilized receptors. This finding is in agreement with the relatively modest inhibition by NaCl of high-affinity [^{3}H]etorphine binding to brain membranes (32). In contrast, NaCl produced a marked inhibition of [^{3}H]DHM binding (50 μM NaCl reduced [^{3}H]DHM binding by 55% relative to the control in the absence of added salts).

Preliminary characterization of solubilized brain opiate receptors. The sizes of prelabeled solubilized brain opiate receptors were estimated by molecular exclusion chromatography (Fig. 6). Radioactivity from the prelabeled [^{3}H]etorphine-receptor complex (Fig. 6A) chromatographed as two broad elution peaks. The first occurred at a position corresponding to a protein species of Stokes radius 63 Å, by comparison with standard globular proteins of known sizes (Fig. 6B), and the second at the salt peak (as determined by the elution of phenol red). The molecular weight for a spherical macromolecule of 63 Å Stokes radius is approximately 350,000. This size estimate agrees with the values previously determined for the covalently bound [^{3}H]enkephalin macromolecule complex and noncovalent [^{3}H]etorphine complex, using Brij 36T to solubilized opiate receptors (34, 35). The second peak may represent free etorphine or a small protein of ≤20 Å. Results identical with those shown in Fig. 6A were obtained when the CHAPS concentration was increased from 1 mM to 2 mM. A mixture of [^{3}H]etorphine (2 nM) and CHAPS (1 mM) eluted close to the salt peak. Together, these results indicate that the [^{3}H]etorphine is most likely not binding to detergent micelles.

The μ opiate receptor (nonlabeled), as measured by specific binding of [^{3}H]DHM, migrated as two peaks (Fig. 7), one at an elution position 18 ml after the void peak and one at approximately the position of the salt peak. The elution profiles of these corresponded to macromolecules of Stokes radii 70 and ≤20 Å. When a mixture of [^{3}H]DHM (1 nM) and CHAPS (1 mM) was applied to this column, a single peak of radioactivity was observed at the salt peak. The elution positions of the major peak of [^{3}H]bremazocine binding activity eluted just after, and was well separated from, the major peak of [^{3}H]DHM binding. In eight independent experiments, this [^{3}H]bremazocine binding activity peak eluted at a position 20 ± 3 ml after the major peak of receptor activity. A secondary peak of κ binding activity coeluted with the secondary peak of μ binding activity. The two peaks of [^{3}H]bremazocine binding eluted at positions corresponding to proteins of 54 and ≤20 Å Stokes radii. Free [^{3}H]bremazocine (1 nM) migrated as a single peak of radioactivity at approximately the salt peak. The major peak of [^{3}H]etorphine binding activity occurred at a position corresponding to a protein of Stokes radius 60 Å, approximately midway between the major peaks of μ and κ

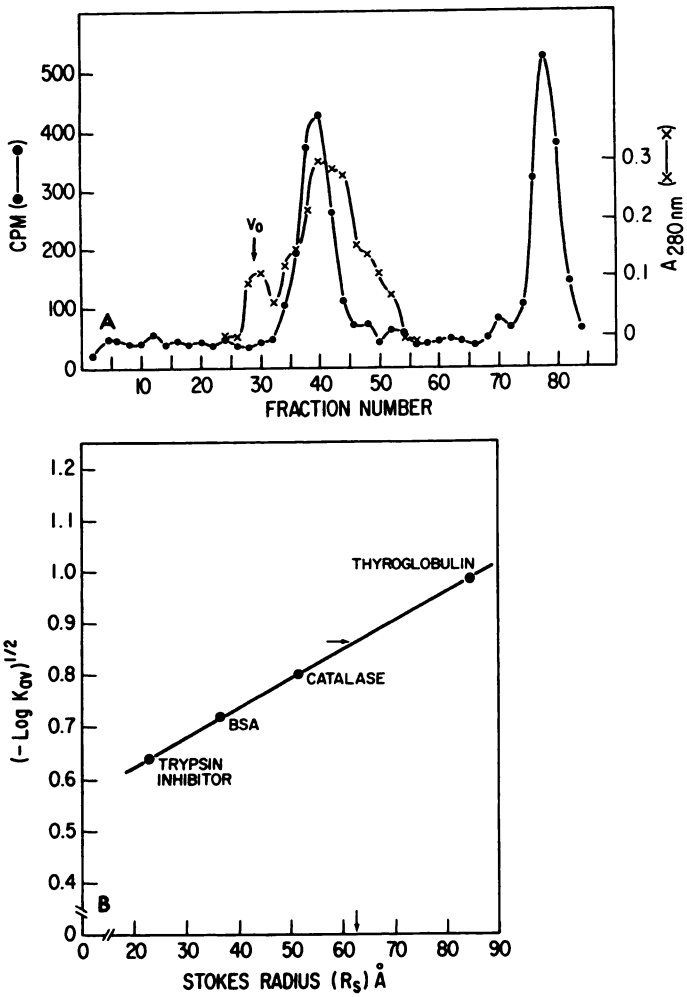

FIG. 6. *Sepharose CL-6B gel filtration of a CHAPS extract of rat brain membranes bound with [^{3}H]etorphine: (A) elution profile of the column and (B) standardization plot for size determination of the prebound [^{3}H]etorphine complex*

Three milliliters of 10 mM CHAPS extract (9 mg of protein), preincubated with 2 nM [^{3}H]etorphine for 1 hr at 4°, was applied to a Sepharose CL-6B column (1 × 85 cm) equilibrated with 0.32 M sucrose/10 mM Tris-HCl (pH 7.4)/1 mM CHAPS at 4°. Two-milliliter aliquots were collected at a flow rate of 10 ml/hr. Aliquots (0.5 ml) were assayed for radioactivity (●) and protein concentration by absorbance at 280 nm (×). In B, standard proteins (10 μl) with known Stokes radii were applied to the column and eluted as above. Data are expressed as $(-\log K_{av})^{1/2}$ versus Stokes radius for each standard, where $K_{av} = (V_e - V_0)/(V_t - V_0)$; V_e, V_0, and V_t refer to the elution volume, void volume, and total volume, respectively (33). The *arrows* refer to the $(-\log K_{av})^{1/2}$ value obtained for the [^{3}H]etorphine-bound opiate receptor and the Stokes radius derived from the plot. V_0 and V_t were 56 ml and 178 ml, respectively. Protein concentrations were monitored by absorbance at 280 nm, and the radiolabeled complex was monitored by radioactivity determination.

binding activity (data not shown). Together, these findings indicate that there may be as many as four protein species of radii 70, 60, 63, 54, and ≤20 Å with opiate binding activity. The presence of low molecular weight opiate binding proteins has also been reported by others (36). Further attempts to characterize the lowest molecular weight species proved difficult because of their extreme lability and inactivation during additional column steps, whereas the highest molecular weight species (putative μ receptor) appears to be relatively stable and has been purified approximately 300-fold by affinity chromatography.[2]

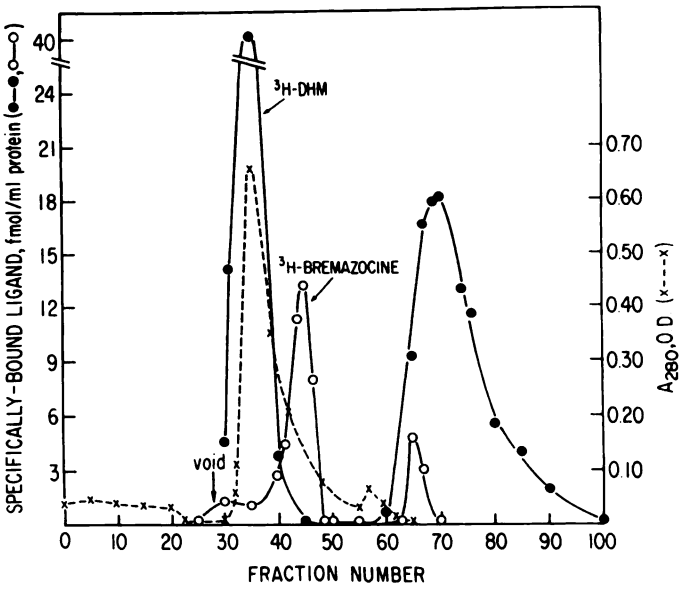

FIG. 7. *Sepharose CL-6B gel filtration of CHAPS extract of rat brain membranes monitored for μ and κ binding activity*

Three milliliters of 10 mM CHAPS extract (9 mg of protein) were applied directly to a Sepharose CL-6B column (1 × 85 cm) and eluted as described in the legend to Fig. 6. Aliquots (100 μl) were assayed in triplicate for specific [^{3}H]DHM (●) or [^{3}H]bremazocine (○) binding in the presence of 40 nM normorphine and 100 nM DADLE (as described under Experimental Procedures) and for protein concentration by absorbance at 280 nm (×). Protein markers were chromatographed as described for Fig. 6. This experiment was replicated five times.

In order to characterize further the putative solubilized κ receptor, the relative potencies of a series of opiates and opioid peptides in displacing [^{3}H]bremazocine binding to CHAPS extract were determined (Table 3). Assays were carried out in the presence of normorphine and DADLE in order to direct [^{3}H]bremazocine binding to κ receptors (17, 18, 30). Data are shown together with those obtained with brain membranes. Of note, only the κ ligands bremazocine and ethylketocyclazocine exhibited high affinities (IC_{50} values of 4 and 10 nM, respectively) for this site.

The pI of the [^{3}H]etorphine-receptor complex was estimated by liquid isoelectric focusing (Fig. 8). The major peak of brain membrane protein, as determined quantitatively by the method of Lowry *et al.* (27) and as visualized directly by turbidity, migrated as a single broad peak to a position corresponding to pH 4. Faintly turbid bands appeared at this migration position by 12–15 hr of focusing; these appeared to aggregate into a single broad peak of turbidity by 40 hr of focusing. This finding suggests that many of the membrane proteins migrated to their final elution position within a few hours and subsequently aggregated, possibly as a result of the acidic environment. Radioactivity from the prelabeled [^{3}H]etorphine-receptor complex chromatographed as two peaks; the first comigrated with the major protein peak at a position corresponding to a pI of approximately pH 4. The second peak eluted at approximately pH 9 and

[2] R. M. Maneckjee, R. S. Zukin, and S. Archer, in preparation.

TABLE 3

Relative potencies of opiates in displacing [^{3}H]bremazocine binding to solubilized κ opiate receptors

Whole rat brain minus cerebellum was used to prepare PEG-purified CHAPS extract, as described under Experimental Procedures. Aliquots of solubilized receptors (0.5 ml, 150 μg of protein) were incubated in triplicate at 37° for 20 min with 2 nM [^{3}H]bremazocine (24,000 cpm), 40 nM normorphine, 100 nM DADLE (κ receptor assay conditions), and nonradioactive opiates at eight concentrations. One hundred percent binding is bound [^{3}H]bremazocine in the presence of normorphine (40 nM), DADLE (100 nM), and cyclazocine (10 μM). Samples were precipitated, filtered, and assayed as described under Experimental Procedures. Log-probit plots were constructed, and IC_{50} values determined by linear regression analysis of the data. Values reported are the means ± standard error of the mean of three independent determinations.

Aliquots of whole brain homogenates (1.0 ml, 0.75 mg of protein), prepared as previously described (16), were incubated in triplicate at 37° for 20 min with 2 nM [^{3}H]bremazocine, 40 nM normorphine, and 100 nM DADLE and indicated nonradioactive opiates at eight concentrations. Specific binding is as defined above. Samples were filtered and counted as described (16). IC_{50} values were determined by linear regression analysis of log-probit plots. Values reported are the means ± standard error of the mean of three independent determinations.

Drug	CHAPS extract		Brain membranes	
	IC_{50}	Relative potency	IC_{50}	Relative potency
	nM		*nM*	
Bremazocine	4.3 ± 0.4		6.3 ± 1.9	
Ethylketocyclazocine	10 ± 1.5	0.43	10.4 ± 0.6	0.61
Naloxone	5000 ± 112	0.0008	10,000	0.0006
D-Ala^2,*N-Me*-Phe^4-Met(*O*)-ol^5-enkephalin	1000 ± 95	0.0004	1,560 ± 500	0.004
Dihydromorphine	375 ± 12	0.011	1,225 ± 275	0.005
DADLE	42,000 ± 1,100	0.0001	6,800 ± 410	0.0009
Dextrorphan	16,000 ± 1,500	0.0002	21,000 ± 4,000	0.0003

presumably corresponds to free etorphine. These data indicate that the opiate receptor is an acidic protein, much like the majority of the brain membrane proteins.

DISCUSSION

Opiate narcotic agonists and antagonists have been shown to bind stereospecifically, with high affinity, and reversibly to CHAPS-solubilized rat brain membranes. [^{3}H]Etorphine, [^{3}H]DHM, (μ ligand), and [^{3}H]bremazocine (κ, μ, and δ ligands) each exhibited binding to an apparent single class of high affinity (K_D = 0.8–2 nM) sites. [^{3}H]Bremazocine binding in the presence of normorphine and DADLE occurred to 25 fmole sites per milligram of protein with K_D = 0.6 nM; these sites are tentatively identified as κ receptors based on the known pharmacological receptor selectivity of this ligand in rat brain tissue (29) and its binding specificity under these conditions (17, 18, 30). [^{3}H]DADLE binding was essentially undetectable in this preparation under these binding conditions (10 mM Tris-HCl buffer, pH 7.4), but could be demonstrated in the presence of $MnCl_2$, GTP, and NaCl.

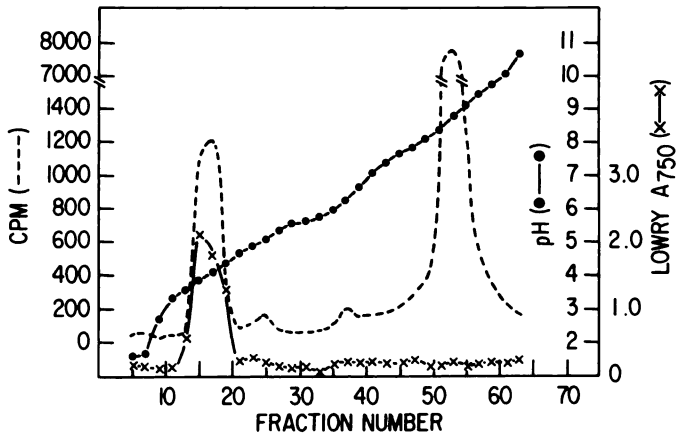

FIG. 8. *Liquid isoelectricfocusing of [^{3}H]etorphine-bound CHAPS extract from rat brain membranes*

A CHAPS-solubilized preparation preincubated with [^{3}H]etorphine (2 nM) for 1 hr at 4° and washed two times prior to addition of CHAPS (see Experimental Procedures) was fractionated by liquid isoelectric-focusing with a 100-ml electrofocusing column equipped with a circulating water jacket at 20°. A stabilizing gradient of 0–50% sucrose was used with carrier ampholytes at a concentration of 1% in the pH range 3–10. The sample (5 ml) was introduced midway into the column as the gradient was being made. Electrofocusing was performed at 1000 V for 40 hr. Two-milliliter fractions were collected at a flow rate of 2 ml/hr.

Many of the properties of the solubilized opiate binding component(s) are similar to those of brain membrane-bound receptors (37). First, opiate binding to CHAPS extract was of high affinity. Second, opiate binding was markedly inhibited by trypsin, by proteinase K by heat, and by NEM, findings which suggest that the sites are proteinaceous. Third, opiate agonist (but not antagonist) binding to CHAPS extract was inhibited by guanyl nucleotides and (in case of [^{3}H]DHM) by sodium. This finding is of particular interest in light of the implication that the solubilized receptors retain their coupling to a guanyl nucleotide binding protein. By analogy to muscarine and dopamine receptors, which are also negatively modulated by guanyl nucleotides and are so coupled, this finding may indicate that opiate receptors retain an important physiological activity in the soluble state. Inhibition of opiate agonist and antagonist binding to solubilized opiate receptors from NG-108-15 cells by guanyl nucleotides had been reported (38). Fourth, the relative potencies of a series of alkaloid opiates in displacing [^{3}H]etorphine binding were very similar to those determined for rat brain homogenates (9, 37). Finally, the relative proportions of the μ and κ receptor subtypes after solubilization (1.5:1) agrees with that previously found for rat brain membranes (17).

Two findings suggested that the opiate binding components present in the CHAPS extract are in a soluble form. First, these components remain in the supernatant following centrifugation at 100,000 × *g* for 90 min. This is the standard criterion for solubilization. Second, the

[^{3}H]etorphine-receptor complex as well as the μ and κ receptor activities migrate distinctly after the protein void peak of the Sepharose CL-6B column. Insoluble particles would be expected to migrate with the protein void peak (38) and be thereby separated from solubilized membrane proteins. The isoelectric focusing results indicate that the solubilized opiate receptors do aggregate at acidic pH; chromatography was performed in these studies at pH 7.4.

That the peak of radioactivity in Fig. 6A is due to [^{3}H] etorphine-receptor complex and not detergent micelles is indicated by several findings. First, the peak of radioactivity observed when receptors are prelabeled with [^{3}H] etorphine (Fig. 6A) is coincident with the peak of receptor activity observed when the nonlabeled CHAPS extract is applied directly to the column and assayed for binding activity. It is of interest that both peaks are relatively broad and may represent binding of [^{3}H]etorphine to multiple opiate receptor subtypes. Second, the ^{3}H-labeled ligand-detergent complexes elute at the salt peak of the column (determined by the elution position of phenol red). Third, increasing the detergent concentration of the eluting buffer from 1 mM to 2 mM does not alter the elution position of the [^{3}H]etorphine-macromolecule complex (Fig. 6A). Fourth, the critical micellar concentration for CHAPS has been determined to be between 4 mM and 6 mM (39); elution from the Sepharose 6B-CL column (Figs. 6 and 7) was routinely performed using 1 mM CHAPS.

Methods to solubilize opiate receptors from the neuroblastoma-glioma hybrid cell line NG-108-15 (23) (a tissue bearing only the δ subtype) from toad brain (21) (a source of "pure" κ receptors) and from mammalian brain (22) have been described. Opiate binding to the solubilized species was in each case shown to be stereospecific and saturable, and to exhibit the characteristics of a protein of high affinity. Solubilization of the δ receptor from the neuronal cell line was achieved by using the zwitterionic detergent CHAPS. The solubilized detergent-receptor complex had a Stokes radius of 70 Å as determined by gel filtration. Solubilization of the κ receptor from toad brain was achieved by using the detergent digitonin (21); solubilization of opiate receptors from mammalian brain could be achieved only using digitonin in consort with 1 M NaCl (22). This latter method precluded the binding of opiate agonists to the solubilized brain extract. Other reports of opiate receptor solubilization have also been presented (40, 41). In the present study, we used CHAPS to solubilize opiate receptors from rat brain, a source of heterogeneous opiate receptor subtypes, with a high signal-to-noise ratio (the percentage of specific binding ranges from 50 to 85) and relatively high yields (17%–55%). Thus, CHAPS appears to be a particularly useful detergent for the solubilization of brain opiate receptors. Its advantage relative to other detergents may be due to its zwitterionic function and possibly also to its sulfate moiety, which may serve to stabilize the soluble receptors. In this regard, we have also obtained relatively good yields with taurcholate and deoxytaurcholate, which are also sulfate-bearing detergents. The present study has provided a preliminary characterization of the molecular properties of μ and κ receptors.

Several molecular models could explain the observed differences in molecular properties of the μ and κ binding components. First, these may be distinct proteins of differing molecular weights. Second, these may represent different aggregational states of the same subunit protein. The binding of [^{3}H]DHM and [^{3}H]bremazocine to a common smaller molecular weight species is consistent with this model. Third, the two species may represent a single protein in various configurations as, for example, an uncoupled state and a guanyl nucleotide binding protein-coupled state. It is less likely that the receptors represent different ligand-induced conformational states of the same protein, as their differing migration positions were observed for the case of the "unbound" receptors (Fig. 7). Recent findings that (a) κ receptors exhibit a unique brain distribution unlike that of μ, δ, or κ receptors (18) and that (b) dynorphin or one of its fragments may function as an endogenous κ ligand (42, 43) lend support to the concept of a distinct κ receptor system. Further purification of solubilized brain opiate receptors and their visualization on sodium dodecyl sulfate/polyacrylamide gel electrophoresis would be expected to distinguish among these models.

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