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RESOLUTION IN THE RECEPTOR BINDING OF PUTATIVE μ AND κ OPIATES

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Summary

Conditions for the stereospecific binding of ^{3}H-ethylketocyclazocine and ^{3}H-etorphine were established in membranes from rat and pigeon brain. In displacing the specific binding of the radiolabeled ligands, putative μ and κ opiates displayed different sensitivity toward sodium. In membranes from both species, the ratio of the sodium responses exhibited by a given drug in displacing ^{3}H-ethylketocyclazocine and ^{3}H-etorphine, respectively, ("double sodium ratio" = DSR) distinguished between μ and κ opiates. Compounds characterized on the basis of their pharmacological effects as κ opiates had DSR values between 0.3 and 2.2, regardless of their nature as agonists or antagonists. In contrast, the DSR for μ opiates ranged from 3.4 to 11. In rat brain membranes, UM 1382 (U-50,488, a compound with pronounced κ activity) exhibited a DSR of 0.3, while the corresponding value for morphine was 7.4. Dynorphin-(1-13) had a DSR of 1. Within each of the two groups, the simple sodium ratio continued to serve as an index for the agonist or antagonist property of the tested opiates.

Unequivocally different binding properties of putative μ and κ opiate alkaloids have been particularly difficult to demonstrate in rat brain membranes. In characterizing the binding of radiolabeled ethylketocyclazocine (EKC), a compound with typical κ-like activity in vivo (1), the similarity of the data to those obtained with μ opiates was noted (2,3). In earlier reports it was suggested that the effects of specific opiates could be accounted for by their differential interaction with μ and δ opiate binding sites (3-6). Subsequently, by preferentially inhibiting μ and δ opiate binding with relatively specific ligands, residual sites for κ opiates were revealed (7,8). However, the assertiveness of this approach has been hampered by the lack of ligands with high specificity for the different opiate receptor subtypes. In order to circumvent this problem, the simultaneous computer analysis of displacement curves obtained with unlabeled and tritiated μ, κ and δ opiates in rat brain was carried out (9,10). It was later suggested (11,12) that the possibility of an allosteric interaction between μ and δ opiates may require fitting such data to a more complex binding model than the one employed (10). Strong evidence for receptor heterogeneity in guinea pig brain membranes was provided by the observations that putative κ opiates selectively protected the binding of radiolabeled κ, but not μ or δ ligands from inactivation by phenoxybenzamine (13). However,

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in rat membranes such experiments using N-ethyl maleimide yielded equivocal results (3,14), suggesting species differences in the distribution of opiate receptor subtypes.

A new dimension in characterizing a receptor for κ opiates was introduced by the discovery of dynorphin, a putative endogenous ligand for these recognition sites (14,16). Furthermore, alkaloid opiates with distinct κ-like properties were recently described (17,18). Their availability in radiolabeled form should become a valuable tool in assessing the interaction of these opiates with distinct receptor sites.

In the course of our ongoing work on the receptor binding properties of newly synthesized opiates (1,19-23), we have observed that compounds which exhibited κ-like features in their behavioral evaluation in the Rhesus monkey and pigeon (23), were less sensitive to the effect of sodium present in the binding assay. Other studies have also shown data (24) or have noted (25) lower sodium ratios (26) of putative κ agonists relative to their μ counterparts. In this paper we describe the resolution of putative μ and κ opiates on the basis of their sodium responses displayed in displacing the receptor binding of ^{3}H-EKC and ^{3}H-etorphine in membranes from rat and pigeon brain.

Materials and Methods

Materials. ^{3}H-EKC (15 Ci/mmole) and ^{3}H-etorphine (30 or 51 Ci/mmole) were purchased from New England Nuclear Corp., Boston, MA. and Amersham Corp., Arlington Heights, IL, respectively. The purity of these compounds was ascertained by thin-layer chromatography on silicagel G plates (Eastman-Kodak Co., Rochester, NY) using two solvent systems recommended by the manufacturers. Radiochemical purity was in all cases higher than 97%. Dynorphin-(1-13) was kindly provided by Dr. H. Akil from the University of Michigan. The unlabeled opiates were obtained through the Drug Abuse Basic Research Center at the University of Michigan.

The coded compounds included in this study were investigated as part of the preclinical evaluation of new opiates at the University of Michigan. The following brief characterization is based on evidence obtained primarily in behavioral experiments and with smooth muscle preparations (1). UM 909, UM 911, UM 1070, and UM 1071-R are N-furyl-substituted benzomorphans with κ agonist activity (1,27). UM 1071-S is the pharmacologically inactive isomer of UM 1071-R. UM 1382 (U-50,488) (trans-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide) and the structurally related UM 1345 are prototypes of a recently introduced novel series of opioids with demonstrated κ agonist activity (18,28). The oripavine derivative UM 928 is structurally related to etorphine but carries a cyclopropylmethyl group as the N-substituent; it had EKC-like discriminative effects in the Rhesus monkey (28). UM 979 is a N-furyl-substituted 5,9-diethyl benzomorphan with antagonist properties in the monkey (29) and in smooth muscle preparations (30). MR 2266 is an N-furyl-substituted 5,9-diethyl benzomorphan antagonist that appears to have equal potency toward μ and κ agonists (31). The benzomorphan bremazocine is structurally similar to ketazocine and was classified as a κ agonist (17).

Membrane preparation (1,32). Male Sprague-Dawley rats weighing 200 g were decapitated and the brains excised at 4°. The cerebrum was dissected and washed in 50 mM Tris-HCl, pH 7.4. The weighed tissue was disrupted for 1 min in 100 volumes of the ice-cold buffer, using a Polytron homogenizer (model PT-10, Brinkman Instruments Inc., Westbury, NY) at power output 6.5. The homogenate was centrifuged at 20,000 x g for 15 min in the cold, and the

obtained pellet was resuspended with the original amount of buffer using a Dounce all-glass homogenizer. Aliquots of this suspension, sufficient for experiments on one given day, were frozen at -70°. Prior to its use, the suspension was thawed, dispersed in a Dounce homogenizer and kept on ice. The protein concentration in the latter preparation was approximately 0.6 mg/ml, as determined according to Lowry et al. (33).

The isolation of membranes from pigeon brain was carried out as described for rat brain. White Carneaux pigeons were decapitated, the brains excised and washed in 50 mM Tris-HCl, pH 7.4. The subsequent steps were those described above.

Binding assay (1,32). The assay mixture in 8 ml polypropylene tubes consisted of 400 μl of membrane suspension, 50 μl of 50 mM Tris-HCl, pH 7.4, or NaCl solution to give a final concentration of 150 mM sodium, and 25 μl of either ^{3}H-etorphine or ^{3}H-EKC. The final volume of the assay was 525 μl. Constant pH during the incubation was ascertained. After incubation for 40 min at 25° (reflecting binding equilibrium), the samples were filtered through glass-fiber disks (Whatman GF/C). Initially, the filters were repeatedly washed by swirling in water and decanting, and were then treated on the filter assembly with water, saturated at room temperature with n-amyl alcohol (34). The filtered samples were quickly washed with ice-cold 50 mM Tris-HCl, pH 7.4, and placed into polyethylene counting vials. After the addition of 1 ml of absolute ethanol followed by 10 ml of xylene-dioxane-naphthalene-based scintillation fluid, the vials were subjected to liquid scintillation counting. The average counting efficiency, determined by the use of ^3H_2O, was 43%. In experiments with dynorphin, special precautions were used to prevent adsorption of this compound onto the tube walls. The assay was carried out in tubes rinsed with a 1% solution of bovine serum albumin in 50 mM Tris-HCl, pH 7.4.

Assessment of specific binding. Stereospecific binding of ^{3}H-EKC and ^{3}H-etorphine was determined by the use of the enantiomers UM 1071-R and UM 1071-S, and levorphanol and dextrorphan, respectively. Receptor-related interaction was defined as the difference between bound ligand obtained in the presence of an appropriate excess of the inactive and active unlabeled isomer, respectively. The resulting "binding window" (32) reflected maximal stereospecific binding. The binding of the radiolabeled ligand in the presence of a given test drug, investigated at five different concentrations, was then expressed as percent of maximal binding. The EC50 values were obtained from log-probit plots, drawn after regression analysis of the binding data, relating inhibition of radiolabeled ligand binding and concentrations of unlabeled opiate. The sodium response ratio for a given compound was expressed as the ratio of the EC50 values obtained in the presence and absence of 150 mM NaCl. The "double sodium ratio" (DSR) was calculated by dividing the sodium ratio determined in displacing ^{3}H-EKC by the sodium ratio obtained with ^{3}H-etorphine binding.

Results

Under the assay conditions described in Materials and Methods, equilibrium in the binding of ^{3}H-EKC (0.5 nM) and ^{3}H-etorphine (0.5 nM or 3 nM) was reached within 40 min. In addition to the data shown for ^{3}H-etorphine in rat brain (Figure 1A) and for ^{3}H-EKC in pigeon brain (Figure 1B), "binding windows" were established for ^{3}H-etorphine in pigeon brain, and for ^{3}H-EKC in rat brain. The concentrations of the displacing enantiomers were selected on the basis of the obtained "binding windows" (Fig. 1). Specific binding of both radiolabeled ligands (0.5 nM) in rat and pigeon brain membranes was determined with 100 nM UM 1071-R/UM 1071-S and levorphanol/dextrorphan,

respectively. In experiments with 3 nM ^{3}H-etorphine, the concentration of levorphanol and dextrorphan was 600 nM. Maximal stereospecific binding of 0.5 nM ^{3}H-etorphine was 82% (rat) and 77% (pigeon), expressed as percent of total radiolabeled ligand-membrane interaction. For the binding of 0.5 nM ^{3}H-EKC these values were 92% (rat) and 93% (pigeon). The validity of using UM 1071-R and UM 1071-S as enantiomers to define the stereospecific binding of ^{3}H-EKC was investigated by comparing the upper limit of the "binding window" to that obtained with the dextrorotatory isomer of EKC. These control experiments yielded similar displacement patterns, i.e., the same width of the "binding window". The use of the UM 1071 enantiomers, rather than those of EKC, became necessary due to the unavailability of (-)-EKC.

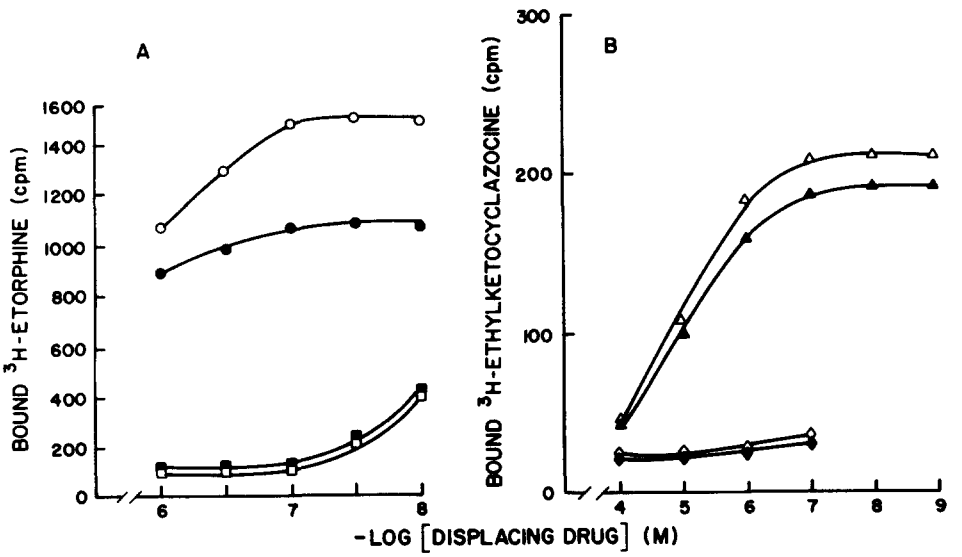

FIG. 1

Displacement of ^{3}H-etorphine and ^{3}H-EKC by pharmacologically active and inactive enantiomers. Aliquots of brain membranes from the rat (Figure 1A) and pigeon (Figure 1B) were incubated as described under Materials and Methods with 0.5 nM ^{3}H-etorphine (Figure 1A), and 0.5 nM ^{3}H-EKC (Figure 1B) in the absence (open symbols) and presence (closed symbols) of NaCl, and in the presence of increasing concentrations of levorphanol (□ ■) and dextrorphan (○ ●) (Figure 1A), and UM 1071-R (◇ ◆) and UM 1071-S (△ ▲) (Figure 1B). Separation of bound opiate, and the determination of radioactivity was as described in the text. The depicted curves were plotted using mean values of data obtained in four experiments, each run in duplicates. The standard deviation around the mean was less than 5%.

TABLE I

Potency of different opiates in displacing the specific binding of ^{3}H-ethylketocyclazocine (EKC) and ^{3}H-etorphine (ET) in brain membranes

Compound	EC50 VALUES(nM)[a]							
	Rat				Pigeon			
	EKC		ET		EKC		ET	
	(-Na)	(+Na)	(-Na)	(+Na)	(-Na)	(+Na)	(-Na)	(+Na)
Putative μ-opiates								
Morphine	1.77	31.0	60.2(14.0)[b]	142(23.6)	24.8	639	184	430
Levorphanol	0.54	2.76	15.4	21.4	2.94	35.1	19.8	37.5
Naltrexone	0.45	0.52	8.57(2.53)	2.27(0.87)	1.33	1.33	5.6	1.35
Putative κ-opiates								
Bremazocine	(0.68)	(1.03)	(1.89)	(1.42)	-	-	-	-
Buprenorphine	(1.95)	(1.54)	(2.14)	(1.18)	-	-	-	-
EKC	2.07	4.24	19.5(5.22)	19.3(6.6)	3.46	7.03	10.4	9.74
Ketazocine	3.8E	10.6	45.7(10.7)	63.1(14.1)	6.46	17.1	-	-
UM 909	11.8	43.8	87.8	163	25.5	98.3	-	-
UM 911	6.40	13.0	45.5(14.6)	93.8(28.3)	8.78	27.1	40.5	86.3
UM 1070	1.51	2.68	16.4	18.0	3.29	5.01	-	-
UM 1071-R	0.37	0.87	4.26(1.14)	4.71(1.55)	1.22	2.64	-	-
UM 1345	(427)	(1835)	(2435)	(4767)	-	-	-	-
UM 1382	(815)	(1068)	(2338)	(9814)	-	-	-	-
UM 928	(0.55)	(0.46)	(1.03)	(0.56)	-	-	-	-
UM 979	2.88	2.71	31.2	20.9	4.46	3.69	-	-
(-) Mr 2266	1.48	1.29	7.54	5.14	1.91	1.54	5.06	3.05
Dynorphin-(1-13)	17.0	21.9	87.7	118	-	-	-	-

[a]Experimental conditions were as described under Materials and Methods. The EC50 value was defined as the concentration which caused 50% inhibition of specific binding of radiolabeled ligand. The drugs were tested at 5 different concentrations, each run in duplicates. The linear relationship between the probit value of binding inhibition and log of drug concentration was established by regression analysis. Shown are mean values of results obtained in 2-6 experiments in which separate samples of the membrane preparation were used. The standard deviation around the mean was in all cases less than 5%.

[b]The numbers in parentheses were obtained in experiments displacing 0.5 nM rather than 3 nM ^{3}H-etorphine.

TABLE II

Resolution of putative μ and κ opiates on the basis of their sodium responses exhibited in opiate receptor binding

Compound	SODIUM RATIO[a] Rat EKC	Rat ET	Pigeon EKC	Pigeon ET	DOUBLE SODIUM RATIO[a] Rat EKC/ET	Pigeon EKC/ET
Putative μ-opiates						
Morphine	17.51	2.36(1.69)[b]	25.77	2.34	7.4(10.4)	11.0
Levorphanol	5.51	1.39	11.94	1.89	3.7	6.3
Naltrexone	1.16	0.26(0.34)	1.00	0.24	4.5(3.4)	4.2
Putative κ-opiates						
Bremazocine	1.51	0.75	-	-	2.0	-
Buprenorphine	0.79	0.55	-	-	1.4	-
EKC	2.05	0.99(1.26)	2.03	0.94	2.1(1.6)	2.1
Ketazocine	2.73	1.38	2.65	-	2.0(2.1)	-
UM 909	3.70	1.86	3.85	-	2.0	-
UM 911	2.03	2.06(1.94)	3.09	2.13	1.0(1.0)	1.4
UM 1070	1.77	1.10	1.52	-	1.6	-
UM 1071-R	2.35	1.11(1.36)	2.16	-	2.1(1.7)	-
UM 1345	4.30	1.96	-	-	2.2	-
UM 1382	1.31	4.20	-	-	0.3	-
UM 928	0.84	0.54	-	-	1.6	-
UM 979	0.94	0.67	0.83	-	1.6	-
(-) MR 2266	0.87	0.67	0.81	0.60	1.3	1.3
Dynorphin-(1-13)	1.29	1.35	-	-	1.0	-

[a]The data in the first four columns respresent the ratios of the EC50 values (Table I) obtained in the presence and absence of sodium (sodium ratios). Listed in the fifth and sixth columns are the ratios of the sodium ratios obtained in displacing the specific binding of ^{3}H-ethylketocyclazocine (EKC) and ^{3}H-etorphine (ET) in rat and pigeon brain membranes, respectively.

[b]The numbers in parantheses were obtained in experiments displacing 0.5 nM rather than 3 nM ^{3}H-etorphine.

The concentration of ^{3}H-etorphine in the assay was either 0.5 nM or 3 nM. Intially, the experiments were carried out with the higher concentration of ^{3}H-etorphine, then available at the relatively low specific radioactivity of 30 Ci/mmole. Subsequently, the concentration of this ligand was decreased to 0.5 nM, thus approximating its K_D. The K_D values for the high affinity binding component of ^{3}H-etorphine and ^{3}H-EKC in membranes from rat cerebrum were 0.11 nM and 0.58 nM, respectively (S.V. Fischel and F. Medzihradsky, unpublished observations). There was little difference in the corresponding sodium ratios obtained with either 0.5 nM or 3.0 nM ^{3}H-etorphine (Table II), although, as expected, the EC50's obtained at the lower concentration of radiolabeled ligand were decreased (Table I).

The obtained EC50's ranged from subnanomolar to micromolar values (Table I). In both rat and pigeon membranes, the differences in sodium ratios displayed by agonist and antagonists were large for μ opiates, and were particularly pronounced in displacing ^{3}H-EKC (Table II). If the sodium ratios obtained in displacing ^{3}H-EKC were divided by the sodium ratios determined in assays using ^{3}H-etorphine, the values of the resulting "double sodium ratio" (DSR) resolved the tested opiates into two distinct groups (Table II). Opiates with recognized μ-like activity had DSR's higher than those of putative κ opiates, regardless of their agonist or antagonist property. For example, in membranes from both species, morphine and naltrexone had distinctly different DSR's, but the value for the antagonist was still significantly higher than the DSR of any putative κ opiate (Table II). Within the κ group, the DSR, in contrast to the simple sodium ratio, did not differentiate between agonists and antagonists. The obtained resolution was independent of the binding affinity to opiate receptor (EC50 values) of a tested compound. E.g., both EKC and UM 1382, with EC50's in the low nanomolar and micromolar range, respectively, were classified as κ opiates on the basis of their DSR values. The weak binding affinity of UM 1382 (U-50,488) corresponded to its low potency exhibited in behavioral tests (28).

Discussion

The displacement of bound radiolabeled ligand is a widely used approach to assess the receptor binding affinity of unlabeled compounds. Although relatively simple in its application, the method carries a liability for erroneous interpretations of the resulting data (36). In implementing the study described here, strong emphasis was placed on avoiding artifacts due to the use of inappropriate experimental conditions. The radiochemical purity of the tritiated opiates was established and monitored throughout the study. In order to ascertain the proper relationship between the determined EC50's and the K_i of the tested compounds, the concentrations of the radiolabeled ligands were selected by considering their respective K_D's in the membrane preparations studied. In order to satisfy the kinetic requirements, some of the experiments with ^{3}H-etorphine were repeated at a lower concentration of the tritiated opiate (Table I). The effect of radiolabeled ligand concentration on EC50's is illustrated by the considerably higher values obtained in displacing 3 nM relative to 0.5 nM ^{3}H-etorphine (Table I). The K_D value was also of importance in establishing a kinetically valid filtration time to avoid possible dissociation of bound ligand in the course of the process. At a K_D of approxiamtely 1 nM the allowable separation time is 1.7 min (36), a condition fulfilled in our study. The time dependence of the binding equilibria, in the presence and absence of sodium, was investigated and the appropriate conditions then adopted. Specific binding was determined by the principle of stereospecificity in the interaction of ligands with the opiate receptor (35). We have carried out a detailed analysis of the methodological aspects of this principle, and have described experimental requirements and

potential artifacts in its application (32). The use of levorphanol and dextrorphan, and UM 1071-R and UM 1071-S to assess the stereospecific binding of ^{3}H-etorphine and ^{3}H-EKC, respectively, was based on the property of these compounds displayed in behavioral tests (1,23), and in in vitro systems such as smooth muscle preparations and receptor binding (37).

Behavioral evaluation represented the primary basis on which the tentative classification of the opiates listed in Tables I and II was carried out. Therefore, discrepancies between the behavioral responses and molecular properties of a given opiate, e.g., its response to sodium in receptor binding, were likely to be reflected as anomalies within the implemented classification of the compounds. However, the agreement between the described molecular property ("double sodium ratio") of the tested opiates, including both agonists and antagonists, and their effects displayed in behavioral test systems (1,23,27) was impressive (Table II). The grouping of the tested compounds into μ and κ opiates was reinforced by the similarity of such resolution achieved in brain membranes from two species. Furthermore, these results were obtained with compounds displaying a wide range of affinities in binding to opiate receptor (Table I). Dynorphin-(1-13), the putative endogenous ligand for the κ receptor (15,16), and UM 1382 a novel analgesic with pronounced κ activity (18,28), exhibited DSR values characteristic for κ opiates. With respect to buprenorphine the results were equivocal. This compound was shown to behave like a partial μ agonist (38), but in our study it responded as a κ opiate (Table II). In a recent study with rat brain membranes, buprenorphine exhibited high binding affinity for all of the opiate receptor subtypes labeled by ^{3}H-diprenorphine, an opiate with low specificity (39). In this conjunction, the importance of species difference in characterizing the heterogeneity of opiate receptor should be emphasized (40). The behavioral tests considered in classifying the drugs in this study were carried out in the Rhesus monkey and Carneaux pigeon, and little information is available on corresponding responses in the rat.

Early data on the opiate receptor binding of various narcotic drugs showed that compounds later identified as putative κ agonists had sodium ratios indicative of mixed agonist-antagonist of the μ type (26). Subsequently, it was concluded that the lower sodium ratios for κ agonists, relative to those of their μ counterparts, are not due to an antagonist component of these compounds (13). Instead, it was suggested that the observed sodium ratios of κ opiates were the consequence of decreased sensitivity toward sodium in binding to receptor. Within our study on the mechanisms underlying the heterogeneity of ligand-opiate receptor interaction, we have recently obtained evidence for the differential effects of μ and κ opiates on the dissociation from receptor of opiate ligands (S.V. Fischel and F. Medzihradsky, unpublished observations). These findings indicate a degree of discrimination between μ and κ opiates occuring within the high affinity opiate binding sites in the presence of sodium. Notwithstanding the ongoing investigations on involved mechanisms, the results shown in this paper describe a convenient approach for the in vitro identification of μ and κ opiates, thus supplementing their evaluation in vivo.

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