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KAPPA-RECEPTOR SELECTIVE BINDING OF OPIOID LIGANDS WITH A HETEROCYCLIC BICYCLO[3.3.1]NONAN-9-ONE STRUCTURE*

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Previous pharmacological results have suggested that members of the heterocyclic bicyclo[3.3.1]nonan-9-one-like compounds are potent κ-opioid receptor specific agonists. One lead molecule of this series, called *compound 1* (dimethyl 7-methyl-2,4-di-2-pyridyl-3,7-diazabicyclo[3.3.1]nonan-9-one-1,5-dicarboxylate) exhibited high affinity for [^{3}H]ethylketocyclazocine and [^{3}H]U-69,593 binding sites in guinea pig cerebellar membranes which known to be a good source for κ_1 receptors. It was shown by molecular modelling that heterocyclic bicyclo[3.3.1]nonan-9-ones fit very well with the structure of ketazocine, a prototypic κ-selective benzomorphan compound; when compared to the arylacetamide structure of U-69,593, a specific κ_1-receptor agonist, a similar geometry was found with a slightly different distribution of the charges. It is postulated, that the essential structural skeleton involved in the opioid activity is an aryl-propyl-amine element distributed along the N7-C6-C5-C4-aryl bonds.

Keywords: Opioid receptors – κ-opioids – radioligand binding – guinea pig cerebellum

INTRODUCTION

Opioid receptors display heterogeneity, consisting of at least three major types, designated as mu- (μ), delta- (δ) and kappa- (κ) receptors [5]. Each of these receptor types have been cloned and their structures solved [15]. The primary amino acid sequences of the different receptors show about 60–70% homology with each other. Although further multiplicity of all the types (μ_1, μ_2, μ_3, δ_1, δ_2, κ_{1-4} etc.) has been described, the molecular basis of this is not fully understood [22]. The use of highly selective ligands and radioprobes is essential for the sufficient biochemical and pharmacological characterization of the multiple opioid receptor system.

Kappa receptors are recognized by a variety of different chemical structures, including the endogenous opioid peptide dynorphin, its synthetic analogous or shortened fragments. Some of the benzomorphans (ketazocine, cyclazocine, bremazocine), arylacetamides (U-50,488, U-69,593, PD-117,302, CI-977) and other non-peptide ligands, such as tifluadom display also high affinity for κ-receptors [27].

* Dedicated to Professor Maria Wollemann on the occasion of her 80th birthday.

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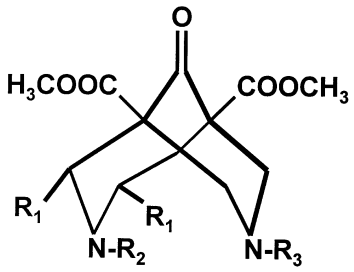

Fig. 1. Structures and systematic names of numbered compounds

1 dimethyl 7-methyl-2,4-di-2-pyridyl-3,7-diazabicyclo[3.3.1]nonan-9-one-1,5-dicarboxylate
R_1 = 2-pyridyl R_2 = H R_3 = methyl

2 dimethyl 7-cyclopropylmethyl-2,4-di-2-pyridyl-3,7-diazabicyclo[3.3.1]nonan-9-one-1,5-dicarboxylate
R_1 = 2-pyridyl R_2 = H R_3 = cyclopropylmethyl

3 dimethyl 3,7-dimethyl-2,4-di-2-chlorophenyl-3,7-diazabicyclo[3.3.1]nonan-9-one-1,5-dicarboxylate
R_1 = 2-chlorophenyl R_2 = methyl R_3 = methyl

Extensive search for novel κ-compounds is of great interest because selective κ-agonists could be strong analgesics devoid of many of the side effects associated with morphine-type (μ-opioid) agonists [20].

In previous systematic studies with heterocyclic bicyclo[3.3.1]nonan-9-ones (compounds *1, 2* and *3* in Fig. 1 and related structures), naloxone-reversible analgesic profile in the tail flick assay, phenylquinone writhing test as well as hot-plate test, inhibition of contractions on the mouse *vas deferens* bioassay and receptor binding characteristics in rat brain membranes have been reported [1, 6, 7, 13, 16, 24, 28]. In the present work we describe selective radioligand binding data for some of these compounds expecting κ-opioid receptor specificity. Binding characteristics are compared to well known reference ligands with different μ-, δ- and κ-receptor selectivity. Structural and physicochemical properties of compounds *1* and *2* were compared with already described κ-agonists (ketocyclazocine and U-69,593) by means of molecular modelling.

MATERIALS AND METHODS

Chemicals

(±)Ethylketocyclazocine methanesulfonate (EKC) was supplied by Sterling Winthrop Research Institute (Rennselaer, NY). U-69,593 = 5–α,7–α,8–α-(–)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl]-benzeneacetamide and U-50,488 = *trans*-3,4-dichloro-N-(methyl)-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide were from the Upjohn Co. (Kalamazoo, MI). All other reagents for

radioligand binding assays were of analytical grade and obtained from SIGMA Chemicals Co. (St. Louis, MO). [^{3}H]naloxone (72 Ci/mmol; [25]) and [^{3}H]Ile^{5,6}deltorphin II (49 Ci/mmol; [21]) were synthesized in the Isotope Laboratory, BRC, Szeged, Hungary. [^{3}H]U-69,593 (44 Ci/mmol) and [^{3}H]DAMGO (59 Ci/mmol) were from Amersham. [^{3}H]EKC (15 Ci/mmol) was purchased from DuPont-New England Nuclear. The derivatives *1–5* were synthesized as previously described [24].

Membrane preparations

Guinea pigs (R9 laboratory strain, both sexes) were used in the opioid receptor binding assays. Cerebellar membranes were prepared as described earlier [3]. Briefly, the cerebella were washed with chilled physiological saline and then homogenized in ten volumes (wt/vol) Tris-HCl buffer A (50 mM, pH 7.4 at 4 °C) with a Braun teflon-glass homogenizer. Homogenate was filtered on four layers of gauze and centrifuged (25,000 × g, 20 minutes, 4 °C). The pellet was resuspended in buffer B (50 mM Tris-HCl, 320 mM sucrose, pH 7.4 at 4 °C) by passing successively through 16 and 21 gauge needles. Membranes were frozen in liquid nitrogen and stored at –70 °C until use.

Radioreceptor binding experiments

Ligand binding assays were performed in duplicate and repeated several times. Frozen membranes were thawed at room temperature and washed in 8 volumes of buffer A by centrifugation (25,000 × g, 20 minutes, 4 °C). Pellets were then resuspended in fresh buffer A to yield a protein concentration of about 0.3 mg/ml and immediately used. Aliquots from the membrane suspensions were added to the assay mixture containing the radioligand and competitors at appropriate concentrations at a final volume of 1 ml. Incubations were started by the addition of the membrane proteins and continued in a shaking water bath until equilibrium was achieved (24 °C, 40 minutes for labelled EKC; 30 °C, 60 minutes for labelled U-69,593; 0 °C, 60 minutes for labelled naloxone and 35 °C, 45 minutes for labelled DAMGO and Ile^{5,6}deltorphin II). Reactions were terminated by rapid filtration on a Brandel M24R cell harvester through Whatman GF/B or GF/C glass fiber filters followed by washing with 3 × 5 ml of ice cold Tris-HCl pH 7.4 buffer. Filters were dried and the bound radioactivity was determined by liquid scintillation counting in a Wallac 1409 (Turku, Finland) spectrometer. Total binding was defined as that measured in the absence of a competing agent. Non-specific binding was determined in the presence of 10 μM unlabelled naloxone and subtracted from the total value to give specific binding. Experimental data from competition experiments were processed using computer-assisted nonlinear regression analysis based on a generalized model of ligand-receptor interactions using GraphPadPrism (version 2.1) software package.

Molecular modelling and ^{1}H-NMR studies

Calculations were based on the free bases. Starting geometries were generated from X-ray data followed by optimization of the conformations by means of force field (MMX2; PCModel Serena Software, Bloomington, Ind., USA). Semiempirical calculations were carried out using MOPAC (Program no. 501, QCPE Bloomington; on an IBM 3084/3081). Molecular graphics studies were carried out on a PS390/microVAX system (Evans and Sutherland/Digital Equipment Co.) using SYBYL software (Tripos Associates, St. Louis, Mo., USA). Molecules were fitted using the fit option as well as the multifit algorithm of SYBYL, which includes an energy minimization of each molecule. Rotational barriers were obtained from data of heats of formation calculated semiempirically (MNDO) for different conformations. The ^{1}H-NMR spectra were recorded using a Varian XL 300 instrument operating at 299.957 MHz. Samples were prepared in a 5 mm tube as ca. 2% solution in DMSO-d_6/D_2O (ratio 1 : 1). Sodium 3-(trimethylsilyl)propane was used as an internal reference at 35 °C the NOE difference spectra were recorded using the Varian program package.

RESULTS

Opioid receptor binding properties of the ligands (Fig. 1) were tested in guinea pig cerebellar membrane fractions [3]. Membranes were labelled with the tritiated opioid antagonist [^{3}H]naloxone, the agonist-antagonist [^{3}H]EKC [3, 27], and with the pure κ-agonist [^{3}H]U-69,593 [18, 19]. Both homologous and heterologous competition assays were performed at binding equilibrium conditions. Representative displacement curves can be shown in Fig. 2, and the results of the competition studies

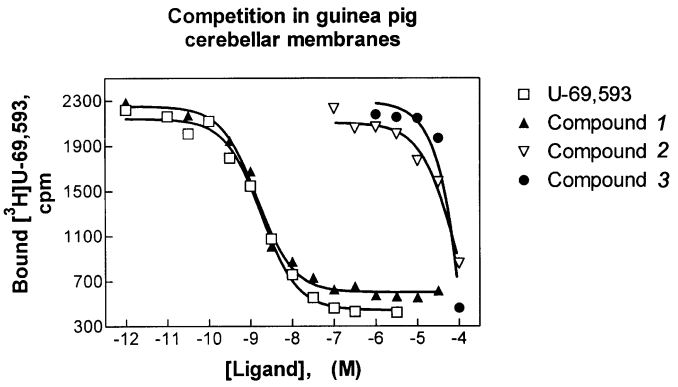

Fig. 2. Membranes from guinea pig cerebella were incubated with [^{3}H]U-69,593 (0.8 nM) in the presence of unlabelled compounds tested for 60 minutes at 30 °C. Curves were analysed by the GraphPadPrism programme (GraphPad Software Inc., San Diego, Ca. USA)

are summarized in Table 1. Naloxone is known as a ligand capable of labelling all opioid receptor types. [^{3}H]EKC is moderately selective for the κ-sites but it selectivity can be improved by the presence of blocking agents for the μ- and δ-receptors. U-69,593 is a κ_1-specific ligand which labels opioid receptors of guinea pig and rat brain membranes. Opioid δ-receptors were measured with the highly selective deltorphin analogue, [^{3}H]Ile^{5,6}deltorphin-II [21]. The type-selective ligands exhibited high affinity in homologous competition experiments, when a labelled ligand was displaced by its unlabelled form. Equilibrium inhibitory constants (K_i values) were in, or under the nanomolar range, indicating the presence of high affinity opioid receptor binding sites in the guinea pig cerebellum.

Compound *1* displaced the κ-selective radioligands tested with much higher affinity than μ- and δ-radioligands in guinea pig cerebellar membranes, which is considered to be a good source of κ_1-receptors. Compound *1* competed for the κ-receptor sites with very high affinity; K_i values were 6.8 and 1.5 nM for [^{3}H]EKC and [^{3}H]U-

Table 1
Equilibrium binding affinities for opioid compounds in guinea pig cerebellar membranes

Labelled ligand/Receptor specificity	Unlabelled competitor ligand	K_i (nM)
[^{3}H]Naloxone	Naloxone	0.73±0.47
General antagonist	*1*	55.8±20.7
	2	158.8±71
[^{3}H]EKC	EKC	2.08±0.46
κ-agonist with μ-antagonist property	*1*	6.8±1.7
	1 with Na^+ ions	34.3[a]±8.6
	1 with GppNHp	29.3[b]±4.1
	2	3304±570
	3	>5000
[^{3}H]U-69,593	U-69,593	0.95±0.2
Selective κ_1-agonist	U-50,488H	1.8±0.3
	1	1.5±0.4
	2	>5000
	3	>5000
[^{3}H]DAMGO	DAMGO	6.3±1.7
μ-specific agonist	U-50,488H	642.0±270
	1	1090.0±346
	2	>5000
[^{3}H]Ile^{5,6}deltorphin II	Ile^{5,6}deltorphin II	0.3±0.12
δ-specific agonist	*1*	337±151

Guinea pig cerebellar membranes were incubated with the appropriate labelled ligand (0.4–1 nM) in the presence of a wide concentration range of unlabelled opioid compounds. Equilibrium homologous and heterologous competition experiments were performed and analysed by computer fitting (GraphPadPrism version 2.1). Values are the mean ± S.E.M. of at least three duplicate experiments.

[a] 100 mM NaCl was present

[b] 100 μM 5'-guanylyl-imidodiphosphate (GppNHp) was present

69,593, respectively. Compounds *2* and *3* displayed much lower affinity in this system, exhibiting K_i values in the micromolar range for both [^{3}H]EKC and [^{3}H]U-69,593 displacement. In the case of compound *1,* the *in vitro* regulation of the binding was examined in the presence of NaCl salt or the stable GTP nucleotide analogue, 5'-guanylyl-imidodiphosphate (GppNHp). In both cases, a significant decrease of affinity was observed, providing biochemical evidence for the agonist property of the compound. As far as the selectivity is concerned, displacement of [^{3}H]DAMGO was also carried out, where compound *1* showed a K_i of 1090 nM, thus the μ/κ selectivity ratio was found to be 600, comparable to that of the reference Upjohn κ-compound, U-50,488H.

Previous studies of the structure of 2,6-diarylsubstituted 3-oxa-7-aza- and 3,7-diaza-bicyclo[3.3.1]nonan-9-ones revealed a chair-chair conformation [2, 17] in the case of the oxygen in 3-position as well as in the case of the nitrogen at that position [8, 10] from both crystals and semiempirical calculations. Thus, we built up the geometry of the diazabicyclo[3.3.1]nonan-9-one from the X-ray analysis data [8] and calculated the energetically favorable conformations by means of AM1. The calculated chair-chair conformation is similar to the geometry found by X-ray analysis [8]. Additionally we found that a chair-boat conformation is energetically less favorable ($\Delta\Delta H = -11.3$ kcal/mol (MM); $\Delta\Delta H = -3.6$ kcal/mol [MNDO]) than the chair-chair conformation. The aryl substituents at C2 and C4 can rotate. Therefore the rotational barrier was calculated from the heats of formation using the force field of PCModel, as well as MNDO and AM1. The results are equivalent with those already reported for the corresponding N,N-dimethyl-diazabicyclo[3.3.1]nonan-9-ones [13]. The energetically favorable conformation is characterized by a dihedral angle (C1,C2,i-C,o-C) of about 90°, which is comparable with the dihedral angle found in solid state.

^{1}H-NMR NOE difference spectra measured in DMSO/D_2O (ratio of 1 : 1) revealed the same geometry in solution indicated by a negative NOE effect between the hydrogens attached to C2/o-phenyl and C4/o-phenyl, respectively. Because the geometries of the heterobicyclic compounds are nearly the same in crystal, solution, and in gaseous state, it is likely, that this conformation is the pharmacologically effective one.

The geometries of the arylacetamide U-69,593 [9] and morphine analogue ketazocine [26] were generated from X-ray analysis data. The most favorable conformations of the compounds (*1,* U-69,593 and ketazocine) were minimized and superimposed with each other, using the aromatic ring and basic N-7 as fitting criteria, since it seems, that the distance between these points plays a crucial role in receptor interaction. The chair-chair conformation of *1* and *2* showed the best fit with ketazocine (rms ~ 0.056). U-69,593 has an extended conformation in the crystal structure which gives a poor fit with ketazocine (or *1*) but can be folded giving a relatively good overlap of the aromatic rings and basic nitrogens. Minimization of *1* as well as U-69,593 against ketazocine, using the MULTIFIT option of the SYBYL program package, also showed that compound *1* fits with ketazocine very well and is energetically more favorable than the fit obtained from U-69,593 and ketazocine.

DISCUSSION

Receptor binding and molecular modelling data for some members of an innovative class of opioid compounds with κ-receptor selectivity has been described in this study. The importance of the κ-specific opioid agonists is that the need for novel, strong analgesics free of the abuse potential and side-effects of morphine-like narcotics is still in the focus of research in medicinal chemistry and experimental pharmacology [20, 27]. Although κ-opioid receptors do not seem to be involved in positive reinforcement processes, chronic treatments with κ-agonists can also lead to the development of tolerance or dependence in animal models [27]. Nevertheless, κ-dependence is characterised as 'mild-dependence' and κ-agonists have definitely lower potency for abuse and addiction than morphine derivatives.

Dynorphins, the endogenous ligands for the κ-receptors possess very good pharmacological and biochemical selectivity, but due to the peptide structure, their chemical stability and bioavailability is limited. The prototypic compounds for the κ-receptors were benzomorphans [11]. Selectivity of these molecules turned out to be not enough thereafter, since interactions with sigma-sites and cross-reactivity with the μ-receptors have frequently been reported. Other important issues against benzomorphans were their sedative, psychotomimetic and dysphoric properties [4, 20, 27, 30], as well as they proved to be mixed κ-agonists and μ-antagonists [12]. A highly selective and potent set of κ-agonists was developed and introduced in the 80s based on a benzacetamide structure [18, 29]. U-50,488H and U-69,593 have successfully been used investigating κ-pharmacology, and in the description of ligand receptor interactions. In addition to analgesia, these selective κ-agonists also produced opioid receptor-mediated sedation, diuresis and corticosteroid elevations. The diazabicyclic skeleton [1, 6, 7, 13, 16, 24, 28] examined in the current study, is structurally rather different from the group of arylacetamide derivatives which are well established as κ-agonists. Several heterocyclic bicyclo[3.3.1]nonan-9-ones were found to have a high affinity to κ-opioid receptors [6, 7], and some of them showed a reasonable κ-agonistic activity in the mouse *vas deferens* test [13]. In addition, these compounds were characterized by long duration of action and high oral bioavailability [14]. Although these compounds bear at least one positive charge under physiological conditions they showed a considerable lipophilicity, indicating the possibility of passing the blood-brain barrier [16].

In guinea pig cerebellar membranes, which is a rich tissue source of κ_1-opioid receptors [3, 23], one of the compounds tested, dimethyl 7-methyl-2,4-di-2-pyridyl-3,7-diazabicyclo[3.3.1]nonan-9-one-1,5-dicarboxylate (compound *1*) was found to be a highly specific κ-ligand. Componds *2* and *3* were much less potent analogues in the selective competition assays (Fig. 2). Agonist property of compound *1* can be predicted by biochemical means from competition binding data obtained in the presence of regulator molecules, such as sodium salt and guanine nucleotides (Table 1). The suggested agonist nature of this compound agrees well with the pharmacological results described previously [1, 13, 28]. Because of the high affinity and selectivity towards the κ-opioid receptors of 2,4-dipyridine substituted 3,7-diazabicy-

clo[3.3.1]nonanone diester having a rather atypical structure (compound *1*) is a lead molecule amongst diazabicyclo[3.3.1]nonanone-type opioid ligands.

It is suggested that the essential structural element contributing to the opioid nature of these compounds is an aryl-propyl-amine distributed along the N7-C6-C5-C4-aryl bonds. Comparison of this part of the molecule *1* with the same moiety in the prototypic benzomorphan EKC and the arylacetamide U-69,593 by means of molecular modelling shows a similar geometry with a slightly different distribution of charges (data not shown). We conclude, that the discussed group of compounds are of theoretical importance, since we have found a unique structure exhibiting κ-opioid activity. In addition, these unusual compounds, which initially appear to be structurally different from the κ-agonists known until now, should be very promising in developing new, highly active κ_1-agonists combined with new pharmacological properties.

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