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κ-Opioid receptors are differentially labeled by arylacetamides and benzomorphans

Daniela E. Rusovici[a], S. Stevens Negus[b], Nancy K. Mello[b], Jean M. Bidlack[a,*]

[a] *Department of Pharmacology and Physiology, School of Medicine and Dentistry, University of Rochester, P.O. Box 711, 601 Elmwood Avenue, Rochester, NY 14642-8711, USA*
[b] *Department of Psychiatry, Harvard Medical School, McLean Hospital, Belmont, MA 02478, USA*

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Abstract

Using Chinese Hamster Ovary cell membranes that stably expressed the human κ-opioid receptor, we investigated the hypothesis that κ_1- and κ_2-opioid receptors, historically defined by their phrmacological selectivity for either arylacetamides or benzomorphans are, in fact, different affinity states or binding sites on the same κ-opioid receptors. Receptor binding studies showed that GTPγS potently inhibited [^{3}H](5α,7α,8β)-(+)-*N*-methyl-*N*-(7-[1-pyrrolidinyl]-1-oxaspiro [4.5]dec-8-yl)-benzeneacetamide (U69,593) binding, compared to virtually no inhibition of [^{3}H]bremazocine binding. Saturation binding experiments showed a three-fold decrease in [^{3}H]U69,593 affinity in the presence of GTPγS, but GTPγS had no effect on [^{3}H]bremazocine affinity. The κ-opioid receptor antagonist nor-binaltorphimine had a four-fold higher affinity for [^{3}H]U69,593-labeled receptors than for [^{3}H]bremazocine-labeled receptors. Functional selectivity studies, measuring the stimulation of [^{35}S]GTPγS agonist-induced binding, showed a significantly higher U69,593-induced G protein-receptor activation in comparison to the stimulation observed with bremazocine. These results suggest that pharmacologically defined 1κ-opioid receptor subtypes may be different affinity states of the same receptor.

Keywords: Benzomorphan; Arylacetamide; Nor-binaltorphimine; κ-Opioid receptor

1. Introduction

The pharmacology of κ-opioid receptor agonists has been the target of a constant scientific inquiry due to increased evidence pointing towards κ-opioid receptor selective-compounds as possible treatments for drug abuse (Mello and Negus, 1998; Negus et al., 1997). Indeed, it has been demonstrated that κ-opioid receptor selective-agonists are capable of reducing the increase in dopamine levels following cocaine administration (Maisonneuve et al., 1994; Collins et al., 2001). In addition, the analgesia induced by κ-opioid receptor selective-agonists was found to be considerably more resistant to the induction of tolerance and withdrawal than analgesia, induced by μ-opioid receptor selective-agonists (Bhargava et al., 1989).

One of the challenges of studying the pharmacology of κ-opioid receptor selective-agonists consists of increased heterogeneity in results for compounds that have been postulated to be selective for κ-opioid receptors and induce similar degrees of analgesia. Several hypotheses were advanced and tested in order to clarify these inconsistencies. Jordan and Devi (1999) demonstrated the existence of homo/heterodimer formation between various opioid receptors, in transfected cell lines, which suggests the putative existence of additional binding pockets formed within the already known opioid receptors. On the other hand, apparent subtypes of κ-opioid receptors were pharmacologically identified in brain tissues of non-human primates and humans (Zukin et al., 1988; Butelman et al., 1998; Caudle et al., 1998; Wollemann et al., 1993). κ_1-Opioid receptors were functionally discovered due to their selective binding to arylacetamides, such as (5α,7α,8β)-(+)-*N*-methyl-*N*-(7-

* Corresponding author. Tel.: +1-585-275-5600; fax: +1-585-273-2652.
E-mail address: jean_bidlack@urmc.rochester.edu (J.M. Bidlack).

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[1-pyrrolidinyl]-1-oxaspiro [4.5]dec-8-yl)-benzeneacetamide (U69,593) (Lahti et al., 1985) while κ_2-opioid receptors were pharmacologically described due to their selective binding to benzomorphans such as bremazocine (Romer et al., 1980). Moreover, κ_1-opioid receptor subtypes were pharmacologically defined as highly selective for the κ_1-opioid antagonist nor-binaltorphimine (Portoghese et al., 1987, 1991), while κ_2-opioid receptor subtypes reportedly showed about a 100-fold lower affinity and selectivity for nor-binaltorphimine (Zukin et al., 1988; Clark et al., 1989). The difference in their molecular structure remains unclear thus far, because only one κ-opioid receptor clone has been found within each species (Raynor et al., 1994). Another possible explanation for the pharmacological heterogeneity of κ-opioid receptors could be the putative existence of different affinity states (i.e., high affinity versus low affinity states) of the same receptor population. Thus, the previously described different subtypes of κ-opioid receptors may actually represent different affinity states or binding sites on the same receptor.

In order to test this hypothesis, we designed a series of experiments that compared and contrasted the pharmacological profiles of two structurally different κ-opioid receptor agonists: U69,593, an arylacetamide, and bremazocine, a benzomorphan. These two ligands have been previously associated with high selectivity for κ_1- and κ_2-opioid receptor subpopulations, respectively (Lahti et al., 1985; Romer et al., 1980). In order to evaluate their binding affinity and selectivity for the different κ-opioid receptor subtypes, competition and saturation binding experiments were designed, in the presence and absence of GTPγS, a stable analog of GTP which is a G-protein uncoupling agent (Ofri et al., 1992). We further compared the efficacies of the two κ-opioid receptor agonists by determining the agonist-stimulated binding of [^{35}S]GTPγS to the alpha subunit of the G protein (Traynor and Nahorski, 1995). The effect of increased GDP concentrations on the agonist-induced G-protein opioid receptor coupling and activation were also studied for both U69,593 and bremazocine. Also, the ability of the selective κ_1-opioid receptor antagonist, nor-binaltorphimine (Marki et al., 2000) to differentiate between the different receptor binding and/or affinity states labeled by either bremazocine or U69,593 was investigated.

2. Materials and methods

Chinese Hamster Ovary cells stably expressing human κ-opioid receptors (hKOR-CHO) (L.Toll, SRI, Palo Alto, CA, USA) were used in all the experiments. These cells were cultured at 37 °C and 10% CO_2, in a Dulbecco's modified Eagle's media (DMEM) enriched with 10% fetal bovine serum and penicillin–streptomycin (10,000 units/ml).

2.1. Chemicals and drugs

U69,593 (Sigma/Research Biochemicals, Natick, MA, USA) was dissolved in 90% ethanol at a stock concentration of 1 mM, and then, was diluted serially to the experimental concentrations. Bremazocine hydrochloride and nor-binaltorphimine hydrochloride (Sigma/Research Biochemicals) were dissolved in double-distilled water. GTPγS and GDP were purchased from Sigma (St. Louis, MO, USA). [^{3}H]U69,593 (64 Ci/mmol), [^{3}H]diprenorphine (50 Ci/mmol) and [^{35}S]GTPγS (1250 Ci/mmol) were purchased from Amersham Biosciences (Piscataway, NJ, USA). [^{3}H]Bremazocine (30 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA). Naloxone was obtained from Sigma/Research Biochemicals. DMEM, fetal bovine serum and penicillin–streptomycin were purchased from Life Technologies (Grand Island, NY, USA). Ecosint A scintillation fluid was purchased from National Diagnostics (Atlanta, GA, USA).

2.2. Competition binding experiments to human hKOR-CHO cell membranes

In order to prepare hKOR-CHO cell membranes, the cells were scraped from 100 mm^2 culture plates. Then, the cells were centrifuged at 200×*g* at 4 °C for 10 min and resuspended in 50 mM Tris–HCl buffer, pH 7.4. The centrifugation step was repeated. The cell pellets were resuspended in 50 mM Tris–HCl buffer, pH 7.4, and centrifuged at 39,000×*g* and 4 °C for 20 min. This step was repeated once, and the resulting membranes were resuspended in 50 mM Tris–HCl, pH 7.4. The protein concentration was determined by the method of Bradford (1976), using bovine serum albumin as the standard.

The IC_{50} values for GTPγS inhibition of receptor ligand binding were determined by competition binding studies in which hKOR-CHO membranes prepared as described above, were incubated for 60 min, at 25 °C with either [^{3}H]U69,593 or [^{3}H]bremazocine, and 12 different concentrations of GTPγS in a final assay volume of 1 ml. Nonspecific binding was measured in the presence of 10 μM naloxone. The samples were filtered through Schleicher & Schuell No. 32 glass fiber filters (Keene, NH, USA) using a Brandel 48-well harvester. The filters were presoaked in 0.25% polyethylenimine freshly prepared for at least 60 min before filtration. After filtration, filters were incubated in 2 ml Ecosint A scintillation fluid overnight and then counted. The experiments were repeated three times with triplicates in each experiment.

2.3. Saturation binding studies to hKOR-CHO membranes

The B_{max} and K_d values of [^{3}H]bremazocine, [^{3}H]U69,593 and [^{3}H]diprenorphine in the absence or presence of 1 μM GTPγS were determined by incubating 15 μg of membrane protein with either [^{3}H]bremazocine or [^{3}H]diprenorphine, or

100 μg of membrane protein with [^{3}H]U69,593, with 8 different concentrations of radioligands at 25 °C for 60 min, in a final assay volume of 1 ml for [^{3}H]U69,593 or 2 ml for [^{3}H]bremazocine and the antagonist [^{3}H]diprenorphine. The remaining steps of the experiment were identical with the ones described above.

2.4. [^{35}S]GTPγS binding studies to hKOR-CHO membranes

Membranes were prepared for [^{35}S]GTPγS binding assays by centrifuging hKOR-CHO cells at 200×*g* and 4 °C for 10 min, followed by resuspension in phosphate-buffered saline buffer (PBS) with 0.04% EDTA, pH 7.4. A second centrifugation at 200×*g* and 4 °C for 10 min was performed followed by subsequent resuspension of the pellet in membrane buffer which contained 50 mM Tris–HCl, 3 mM $MgCl_2$and 1 mM EGTA, pH 7.4. The pellets were further homogenized using a Dounce homogenizer and centrifuged at 39,000×*g* for 20 min at 4 °C. The resulting membrane pellet was resuspended in membrane buffer and recentrifuged as described above. The membranes were finally resuspended in assay buffer which contained 50 mM Tris–HCl, 3 mM $MgCl_2$, 100 mM NaCl and 0.2 mM EGTA, pH 7.4. The protein concentration was determined by the method of Bradford (1976), and then the membranes were aliquoted in cryovials and frozen at −80 °C.

hKOR-CHO membranes were incubated with 12 different concentrations of the compound of interest (i.e., bremazocine, U69,593, GDP, nor-binaltorphimine) in the presence of 0.08 nM [^{35}S]GTPγS, in a final volume of 0.5 ml. Nonspecific binding was measured in the presence of 10 μM GTPγS. Basal activity was determined in the presence of 3 μM GDP. After incubating for 60 min at 30 °C, the membranes were then filtered through Schleicher & Schuell No. 32 glass fiber filters, followed by washing with 3-ml ice-cold 50 mM Tris–HCl, pH 7.5, which was repeated three times. All experiments were repeated at least three times and were performed in triplicate.

2.5. Statistics

Nonlinear regression analysis was used to derive B_{max} and K_d values from Scatchard plots (Ligand program by Munson and Rodbard, 1980). Linear regression analysis (least-squares fit to a logarithm probit equation) was used to calculate IC_{50} values (Tallarida and Murray, 1986). The K_i values were calculated according to the Cheng–Prussoff equation $K_i = IC_{50}/(1+S)$ where S=(concentration of radioligand)/(K_d of radioligand) (Cheng and Prussoff, 1973). Logistic regression analysis was used to compute EC_{50} values based on nonlinear curve fitting of the dose–response curves (SigmaPlot, Jandel Scientific, San Rafael, CA) for the different compounds investigated. Student *t*-tests were computed to calculate the significance of the differences seen with different experiments.

3. Results

3.1. Effect of GTPγS on [^{3}H]bremazocine and [^{3}H]U69,593 binding

To determine if GTPγS altered either [^{3}H]bremazocine or [^{3}H]U69,593 binding to hKOR-CHO membranes, the binding of 0.5 nM [^{3}H]U69,593 or 0.1 nM [^{3}H]bremazocine in the presence of 12 different concentrations of GTPγS was determined. Fig. 1 shows that GTPγS inhibited [^{3}H]U69,593 binding with an IC_{50}value of 550±55 nM, the concentration needed to achieve half of the maximal inhibition (I_{max}=74±3%). GTPγS was relatively ineffective in reducing [^{3}H]bremazocine binding to hKOR receptors. The maximal inhibition of [^{3}H]bremazocine binding was approximately 20%, obtained at 1 μM GTPγS (Fig. 1).

3.2. Saturation binding studies to hKOR-CHO membranes

Table 1 summarizes K_d and B_{max} values for [^{3}H]bremazocine and [^{3}H]U69,593 saturation binding in the presence and absence of 1 μM GTPγS, in comparison to the values obtained for [^{3}H]diprenorphine binding. K_d values for the three compounds were significantly different (one-way analysis of variance (ANOVA), $F(2)=439$, $P<0.01$) with [^{3}H]bremazocine showing the highest affinity, followed by [^{3}H]U69,593, and [^{3}H]diprenorphine. B_{max}values for the three compounds were also significantly different with [^{3}H]diprenorphine labeling the highest number of receptors (i.e., approximately six-fold higher than [^{3}H]U69,593 and two-fold higher than [^{3}H]bremazocine). Additionally, in the presence of 1 μM GTPγS, the apparent affinity of [^{3}H]U69,593 for the κ-opioid receptors was significantly

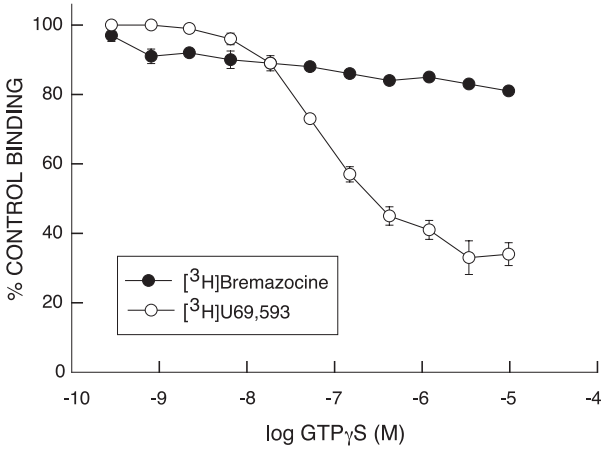

Fig. 1. GTPγS inhibition of 0.1 nM [^{3}H]bremazocine and 0.5 nM [^{3}H]U69,593 binding to human κ-opioid receptors stably transfected on CHO cells.

Table 1
Comparison between the B_{max} and K_d values for [^{3}H]U69,593 and [^{3}H]bremazocine in the absence and presence of GTPγS and the binding of the opioid receptor antagonist [^{3}H]diprenorphine

Experimental condition	B_{max} (fmol/mg protein±S.E.M.)	K_d (nM±S.E.M.)
[^{3}H]U69,593	590±21	0.32±0.035
[^{3}H]U69,593 +1 μM GTPγS	430±13	0.96±0.065[a]
[^{3}H]Bremazocine	1300±92[b]	0.011±0.0004
[^{3}H]Bremazocine +1 μM GTPγS	1200±17	0.012±0.0004
[^{3}H]Diprenorphine	2700±280[b]	2.4±0.1

B_{max} and K_d values for κ-opioid receptor binding of [^{3}H]U69,593 in the absence and presence of 1 μM GTPγS, [^{3}H]bremazocine in the absence or presence of 1 μM GTPγS, and [^{3}H]diprenorphine to hKOR-CHO membranes. The results are expressed as the mean value±S.E.M. from at least three independent experiments performed in triplicate.

[a] $P<0.001$ in comparison to [^{3}H]U69,593 binding alone.

[b] $P<0.05$ in comparison to [^{3}H]U69,593 binding alone.

decreased ($t(2)=8.78$, $P<0.01$), while the receptor number decreased only slightly, when compared to the same parameters obtained in the absence of 1 μM GTPγS. The apparent affinity and receptor number for [^{3}H]bremazocine remained relatively unchanged in the presence of 1 μM GTPγS. The receptor number obtained with [^{3}H]bremazocine was almost three-fold higher than the number of receptors labeled by [^{3}H]U69,593 ($t(2)=9.43$, $P<0.05$).

3.3. Stimulation of [^{35}S]GTPγS binding by U69,593 and bremazocine

The EC_{50} and E_{max} values for U69,593 and bremazocine were measured in a [^{35}S]GTPγS binding assay, in which hKOR-CHO protein membrane was incubated with 12 different concentrations of either U69,593 or bremazocine and [^{35}S]GTPγS. Both κ-opioid receptor agonists were potent agonists in stimulating the G-protein coupling to the κ-opioid receptors, as measured by their ability to stimulate [^{35}S]GTPγS binding. U69,593 (E_{max}=110±3.6%) 3.6%) produced a greater maximal stimulation of [^{35}S]-GTPγS binding than bremazocine (E_{max}=87±2.1%). The EC_{50} values for bremazocine and U69,593 were 6.5±1.6 and 120±18 nM, respectively.

To further investigate the molecular mechanism underlying the coupling and uncoupling of hKOR to the G-protein trimeric unit, and subsequent activation and inactivation of downstream effectors, eight different GDP concentrations were incubated with hKOR-CHO membranes and either 1 μM U69,593 or 1 μM bremazocine, and [^{35}S]GTPγS binding was measured. Fig. 2 summarizes the results of six independent experiments of GDP titration by itself and in the presence of either bremazocine or U69,593. In the absence of agonist stimulation, increasing concentrations of GDP progressively inhibited the basal [^{35}S]GTPγS binding. In the presence of 1 μM bremazocine, increasing concentrations of GDP had an increasing

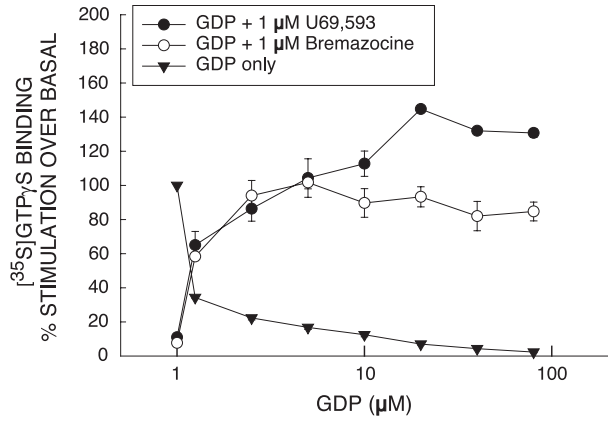

Fig. 2. [^{35}S]GTPγS binding to the human κ-opioid receptor in the presence and absence of 1 μM bremazocine or 1 μM U69,593 and increasing concentrations of GDP.

effect on G protein–receptor coupling reaching an E_{max} value of 109±3% at a concentration of 2.5 μM GDP, and an EC_{50} value of 0.80±0.19 μM. In the presence of 1 μM U69,593, increasing concentrations of GDP also increased [^{35}S]GTPγS binding, reaching an E_{max} value of 140±4.3% at a concentration of 10 μM GDP, and an EC_{50} value of 1.6±0.4 μM.

3.4. Nor-binaltorphimine inhibition of [^{35}S]GTPγS binding induced by κ-opioid receptor agonists

Another way to investigate putative differences between the arylacetamide binding site and the benzomorphan binding site was to determine if the κ-opioid receptor selective antagonist nor-binaltorphimine interacted differently in competing for binding and in inhibiting [^{35}S]GTPγS binding.

The competition binding studies yielded significant differences in the K_i values for nor-binaltorphimine in inhibiting the binding of either [^{3}H]U69,593 or [^{3}H]bremazocine. Nor-binaltorphimine was four-fold more potent in inhibiting [^{3}H]U69,593 binding than [^{3}H]bremazocine binding to the

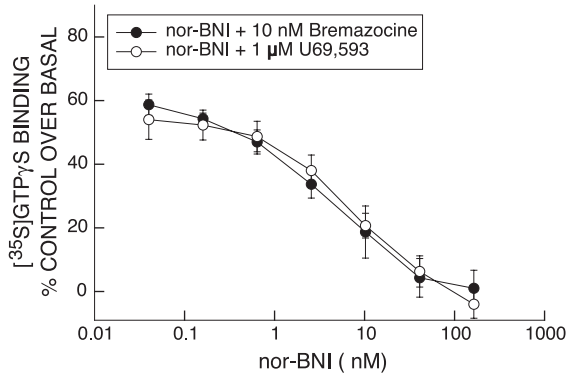

Fig. 3. Inhibition of [^{35}S]GTPγS binding induced by either 1 μM bremazocine or 1 μM U69,593 in the presence of varying concentrations of the κ-opioid receptor antagonist nor-binaltorphimine.

κ-opioid receptor (κ_i values of 0.16±0.004 and 0.66±0.046 nM, respectively (Student *t*-test t(2) = 8.66, $P<0.01$)).

In order to determine the relative ability of nor-binaltorphimine to selectively inhibit U69,593- and bremazocine-stimulated [^{35}S]GTPγS binding, 10 different concentrations of nor-binaltorphimine were incubated with either 1 μM U69,593 or 10 nM bremazocine and hKOR-CHO membrane protein. The concentrations of U69,593 and bremazocine were set at the above values in order to provide a similar amount of agonist stimulation of κ-opioid receptor coupling with the G protein (approximately 60% stimulation). Fig. 3 summarizes the results of nor-binaltorphimine inhibition of [^{35}S]GTPγS binding in the presence of either 1 μM U69,593 or 10 nM bremazocine. No significant differences were found for the inhibition of either U69,593 or bremazocine by nor-binaltorphimine (IC_{50} values of 3.6±0.067 and 2.4±0.51 nM, respectively). The profiles of the displacement curves for bremazocine and U69,593 in the presence of nor-binaltorphimine were very similar. In the presence of 60 nM nor-binaltorphimine, the shift in EC_{50} values was 25-fold for bremazocine and 24-fold for U69,593. Fig. 4 summarizes the effect of 60 nM nor-binaltorphimine on U69,593 and bremazocine stimulated

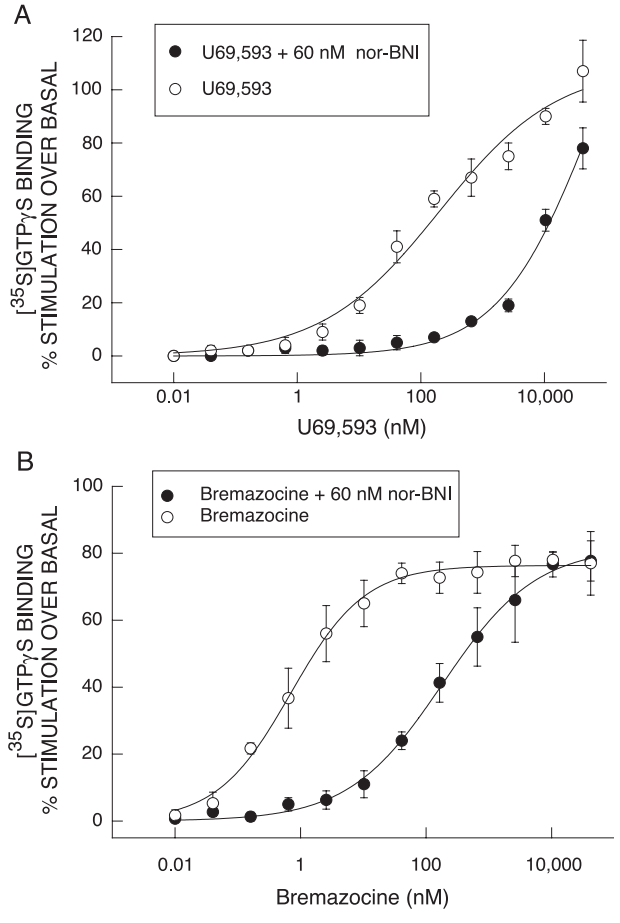

Fig. 4. Titration of U69,593 (A) and bremazocine (B) in stimulating [^{35}S]GTPγS binding to the human κ-opioid receptor in the presence and absence of 60 nM nor-binaltorphimine.

κ-opioid receptor G-protein coupling studies in the presence of 0.08 nM [^{35}S]GTPγS.

4. Discussion

According to the current literature, there may be at least two different κ-opioid receptors functionally defined by their selective binding to either arylacetamides or benzomorphans (Lahti et al., 1985; Romer et al., 1980). This current study was designed to test the hypothesis that the above differentiation in binding profiles between the two compound classes could be attributed to different affinity states of the same κ-opioid receptors.

Our results have shown that GTPγS inhibited the binding of κ-opioid receptors labeled by [^{3}H]U69,593 while having almost no effect on the binding properties of the κ-opioid receptors labeled by [^{3}H]bremazocine. U69, 593 bound with moderate affinity to GTP-sensitive sites, while bremazocine bound with good affinity mostly to GTP-insensitive sites. This finding could further suggest that while U69,593 binds to active, high-affinity G-protein coupled receptors, bremazocine binds to some G-protein coupled opioid receptors but mostly to the G-protein uncoupled κ-opioid receptors. It was previously reported that the dissociation of the receptors from G proteins yields the low affinity form of the ligand occupied receptors (Remmers and Medzihradsky, 1991). Thus, based on this study, bremazocine binds to low affinity opioid receptors.

The saturation binding studies followed by Scatchard analyses yielded significant differences in affinity and receptor densities between bremazocine and U69,593—the two ligands associated with the functional discovery of κ_1- and κ_2-opioid receptors. In the presence of 1 μM GTPγS there was a significant decrease in the affinity of [^{3}H]U69,593 for the opioid receptors, while the receptor density did not change significantly. No GTPγS effect was observed on [^{3}H]bremazocine binding. In light of the fact that GTPγS induces uncoupling of G-protein from the opioid receptors, these results validate again our hypothesis that U69,593 binds to high-affinity G-protein-coupled receptors, binding that is significantly inhibited by the uncoupling action of GTPγS, while bremazocine binds mostly to lower affinity G-protein-uncoupled opioid receptors, binding that is not influenced by the presence of GTPγS.

In order to further investigate if the receptor affinity differences established in our previous binding experiments for bremazocine and U69,593 influenced downstream signal transduction pathways (i.e., G-protein transducer levels), we performed a series of functional experiments using isolated hKOR-CHO membranes. Different concentrations of bremazocine and U69,593 were incubated with hKOR-CHO membranes and the hydrolysis-resistant GTP analog, [^{35}S]GTPγS, in the presence of excess GDP (Hilf et al., 1989; Lorenzen et al., 1993; Traynor and Nahorski, 1995). Our working hypothesis was that the κ-opioid receptor agonist U69,593, by

binding high affinity G-protein coupled receptors, may be able to further mediate a higher affinity GTP binding state translated in an increased efficacy (Kenakin, 1993), when compared to that of bremazocine. The E_{max} values support this hypothesis (Fig. 4A,B). The high-affinity GTP-sensitive receptors, labeled by U69,593, needed a significantly higher agonist concentration to elicit a 50% increase [^{35}S]GTPγS % binding, when compared to the low-affinity GTP-insensitive receptors labeled by [^{3}H]bremazocine. These results may be explained by the putative existence of a concomitant high GDP affinity, along with the enhanced GTP affinity demonstrated earlier, for the G-protein-coupled receptors labeled by [^{3}H]U69,593. This increased GDP affinity would subsequently slow down the displacement of GDP by GTP from the α subunit of the G protein unit (Gilman, 1987). Once the GDP displacement would finally occur (at higher agonist concentrations than expected), GTP would bind the G-α subunit with increasing affinity, resulting in a higher overall G-protein activation for U69,593. As a result, a higher E_{max} value was observed.

Another possible explanation for this apparent inconsistency in the [^{35}S]GTPγS binding results could be provided by the "collision coupling "model first described for the coupling of β-adrenoreceptor and adenylyl cyclase in erythrocytes (Tolkovsky and Levitzki, 1978). According to this model, agonist-activated receptors would function, as mobile catalysts for the G-protein activation, having access to many G proteins per unit time and thus, the level of G-protein activation would be independent of agonist concentration. This model could also explain the results obtained with GDP titration in the presence of set concentrations of bremazocine or U69,593. Although the agonist concentration was maintained constant during the GDP titration, the level of G-protein activation, as measured by [^{35}S]GTPγS binding, increased with increasing GDP concentration. Thus, GDP was acting as a coupling G-protein agent rather than an uncoupling one (Ofri et al., 1992).

The employment of the κ_1-opioid receptor-selective antagonist nor-binaltorphimine in differentiating between bremazocine or U69,593 induced levels of G protein–receptor coupling did not yield any significant results for either compound. The profiles of the displacement curves for bremazocine and U69,593 in the presence of 10 different nor-binaltorphimine concentrations were very similar (Fig. 3). Surprisingly, these results were not decisively influenced by the presence of different receptor affinity states labeled by the two compounds, although in the receptor binding studies, nor-binaltorphimine was four times more effective at displacing [^{3}H]U69,593 from the high-affinity G-protein-coupled receptors, than at displacing [^{3}H]bremazocine from the low-affinity G-protein-uncoupled opioid receptors (Table 1) which is in accordance with previous studies (Marki et al., 2000). If bremazocine and U69,593 were indeed labeling different κ-opioid receptor subtypes (κ_2 and κ_1), which are coupled to the G protein in the same way, nor-binaltorphimine should have exhibited different affinities in blocking activation of G proteins by these two compounds. This result may be explained by the fact that nor-binaltorphimine is acting as an inverse agonist (data not shown), pushing the active/inactive receptor equilibrium towards the inactive side and thus leveling out the differences seen with the receptor binding studies. Alternatively, the presence of excess GDP could have also contributed to even out the differences in affinity seen with tritiated binding experiments, by exerting an uncoupling effect on U69,593-labeled (G protein-coupled) receptors (Ofri et al., 1992).

In summary, our findings support the hypothesis that bremazocine and U69,593 bind to different affinity states of the κ-opioid receptor. U69,593 is clearly labeling guanosine nucleotide-sensitive opioid receptors, while the bremazocine labeled receptors that were insensitive to GTPγS. The present study helps elucidate some aspects of the signal transduction mechanisms underlying agonist efficacy and selectivity for different subtypes of κ-opioid receptors.

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