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Interaction of Bivalent Ligand KDN21 with Heterodimeric δ-κ Opioid Receptors in Human Embryonic Kidney 293 Cells

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ABSTRACT

KDN21 is a bivalent ligand that contains δ and κ opioid antagonist pharmacophores linked through a 21-atom spacer. It has been reported that KDN21 bridges δ and κ receptors that are organized as heterodimers. We have shown previously that when using [^{3}H]diprenorphine as radioligand, KDN21 displayed greatly enhanced affinity in this series for coexpressed δ and κ opioid receptors (CDK). The present study used in vitro expression systems to investigate interactions of members of the KDN series with δ-κ heterodimers through competition binding using selective ligands and the mitogen-activated protein kinase (MAPK) assay. In this regard, the use of the selective radioligands [^{3}H]naltrindole and [^{3}H]norbinaltorphimine (nor-BNI) in competition binding studies revealed that KDN21 has much higher affinity than other KDN members for CDK and bound to CDK more selectively relative to mixed δ and κ opioid receptors or singly expressed δ and κ opioid receptors. Other experiments revealed that the binding of naltrindole to δ opioid receptors could increase the binding of nor-BNI to κ opioid receptors and vice versa, suggesting reciprocal allosteric modulation of receptors in the heterodimer. Regarding the selectivity of KDN21 for phenotypic δ and κ opioid receptors, we investigated the effect of KDN21 on the activation of MAPKs [extracellular signal-regulated kinases 1 and 2 (ERK1/2)] by δ- or κ-selective agonists. KDN21 inhibited the activation of ERK1/2 by [D-Pen^2,D-Pen^5]-enkephalin (δ_1) and bremazocine (κ_2) but had no effect on the activation by deltorphin II (δ_2) and (+)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide (U69593, κ_1). 7-Benzylidenenaltrexone (δ_1) and bremazocine (κ_2) significantly reduced the binding of KDN21 to CDK, whereas naltriben (δ_2) and U69593 produced no such change. Taken together, these data support the idea that the organization of δ and κ receptors as heterodimers gives rise to δ_1 and κ_2 phenotypes.

G-protein–coupled receptors (GPCRs) are typically considered to function as monomers. However, such a model has been challenged by a growing number of studies that have implicated the existence of GPCR dimers/oligomers in cultured cells and in the native state (McLachie et al., 1998; Zawarynski et al., 1998; Zeng and Wess, 1999; George et al., 2000; Gomes et al., 2000; Salahpour et al., 2000; Dean et al., 2001). Opioid receptors belong to rhodopsin receptor family of GPCRs. All three major types (δ, κ, and μ) of opioid receptors share high homology (~60%) and a highly conserved transmembrane domain (Knapp et al., 1995; Dhawan et al., 1996; Waldhoer et al., 2004). Before the development of opioid receptor cDNAs, a number of investigations suggested that opioid receptors physically interact with one another (Erez et al., 1982; Rothman and Westfall, 1982; Portoghese et al., 1986; Porreca et al., 1992; Traynor and Elliott, 1993). With the availability of cDNAs, a great number of studies using in vitro expression systems have afforded more convincing evidence for dimerization/oligomerization of opioid receptors (Jordan and Devi, 1999; George et al., 2000; Gomes et al., 2000; Ramsay et al., 2002). In addition, several recent studies have suggested that heterodimerization of δ and κ opioid receptors may occur in the spinal cord (Garzon et al., 1995; Wessendorf and Dooyema, 2001; Portoghese and Lunzer, 2003). However, it is still not clear how such dimeric opioid receptors are organized and what role they play in signaling. An important step toward this goal would be the develop-

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ABBREVIATIONS: GPCR, G-protein–coupled receptors; CDK, coexpressed δ-κ opioid receptors; MDK, mixed δ and κ opioid receptors which are singly expressed; DOR, δ opioid receptor; KOR, κ opioid receptor; TTBS, Tris-buffered saline/Tween 20; PVDF, polyvinylidene difluoride; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; KDN#, δ-κ opioid antagonist bivalent ligand (K, κ pharmacophore; D, δ pharmacophore; N, antagonist; #, number of atoms in the spacer); BNTX, 7-benzylidenenaltrexone; nor-BNI, norbinaltorphimine; ERK1/2, extracellular signal-regulated protein kinases 1 and 2; MAPK, mitogen-activated protein kinase; U69593, (+)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide; DPDPE, [D-Pen^2,D-Pen^5]-enkephalin; NTB, naltribene.

ment of pharmacological tools that selectively target opioid receptor heterodimers. We have very recently reported on the design and synthesis of a bivalent ligand (KDN21) which may be useful in this regard (Bhushan et al., 2004). KDN21 contains δ and κ opioid receptor antagonist pharmacophores that are connected through a 21-atom spacer (Fig. 1). Intrathecal pharmacological studies in mice and binding studies in HEK293 cells have suggested that KDN21 bridges δ and κ opioid receptors associated as heterodimers (Bhushan et al., 2004). The results of studies with selective agonists and antagonists in vivo are consistent with cooperativity between δ and κ receptors, and it has been proposed that the putative δ_1 and κ_2 opioid receptor subtypes are actually phenotypes (Portoghese and Lunzer, 2003). We reported previously that the affinity of KDN21 for δ and κ opioid receptors coexpressed in HEK293 cells (CDK) was substantially greater relative to other members of the KDN series containing longer or shorter spacers when [^{3}H]diprenorphine was used as radioligand (Bhushan et al., 2004). Although the in vitro binding data were consistent with the in vivo study, it did not identify the binding characteristics of each of the bridged receptors.

Using [^{3}H]naltrindole and [^{3}H]nor-BNI as radioligands in the present study, we identified the receptors bound by KDN21 and confirmed that the 21-atom spacer in the KDN series confers optimal bridging to dimerized δ and κ opioid receptors. Furthermore, we obtained evidence for cooperativity between the receptors in the heterodimer through the facilitated binding of [^{3}H]naltrindole or [^{3}H]nor-BNI in the presence of nor-BNI or naltrindole, respectively. Finally, we demonstrated that KDN21 is selective in its ability to antagonize opioid agonist-induced activation of the mitogen-activated protein kinase (MAPK) signaling cascade. It is significant that KDN21 antagonized only the δ_1- and κ_2-selective agonists that activated MAPKs (ERK1/2), conforming with its pharmacological selectivity in vivo. The present study indicates that KDN21 may become a useful tool in opioid research.

Materials and Methods

Materials. Cell culture and transfection reagents, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, G418 (geneticin), hygromycin, streptomycin, penicillin, trypsin/EDTA, and Lipofectin Reagent were purchased from Invitrogen (Carlsbad, CA). [^{3}H]Diprenorphine (50 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). [^{3}H]Naltrindole (35 Ci/mmol) and [^{3}H]nor-BNI (9.08 Ci/mmol) were supplied by the National Institute on Drug Abuse, National Institutes of Health (Bethesda, MD). U69593, naloxone, and protease inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO). Acrylamide stock (40%) (29:1) was obtained from Fisher Scientific Co. (Hampton, NH). Econo-Safe scintillation cocktail was from Research Products International Corporation (Mount Prospect, IL). Modified Lowry Protein Assay Reagent Kit was purchased from Pierce Chemical (Rockford, IL). Anti-active MAPK pAb and donkey anti-rabbit IgG were purchased from Promega (Madison WI). Full Range Rainbow marker and enhanced cyan fluorescent substrate for Western blotting were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). BNTX; NTB; bivalent ligands KDN15, -20, -21, -22, -23, and -24; and monovalent controls DN21 and KN21 were synthesized in our laboratory. Plasmid cDNAs of δ and κ opioid receptors were kindly provided by Carl Rios and Dr. Lakshmi Devi of New York University (New York, NY). Bremazocine and stable DOR HEK293 cell line were gifts from Dr. Ping Law (University of Minnesota, Minneapolis, MN). Stable KOR HEK293 cell line was kindly provided by Lee-Yuan Liu-Chen (Temple University, Philadelphia, PA).

Cell Culture and Stable Expression of the Receptors in HEK293 Cells. cDNAs encoding rat δ and mouse κ opioid receptors were inserted separately into the mammalian expression vector pcDNA3. The receptors were tagged with different epitopes, respectively: a 10-residue c-Myc epitope (EQKLISEEDL) for the δ opioid receptors and an 8-residue Flag epitope (DYKDDDDK) for the κ opioid receptors. Human embryonic kidney 293 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 unit/ml penicillin under humidified atmosphere in a 10% CO_2 incubator at 37°C. The cells, grown to approximately 50% confluence, were transfected with the expression vectors containing δ or κ opioid receptor cDNA. Transfections were done in serum-free medium using Lipofectin transfection kit according to the supplier's manual (Invitrogen). We used 10 μg of cDNA to transfect the cells of each 100-mm plate. For coexpression, the cells were first transfected with the pcDNA3 vector of δ opioid receptor and then with that of κ opioid receptor. The cells were incubated in transfection medium for 24 h and then changed to be incubated in complete DMEM for another 24 h. The cells were split and transferred into selective medium for culture until the appearance of stable cell colony. The expression level of the receptors was represented by their maximum binding to indicated radioligand (B_{max}). The B_{max} values of the singly expressed DOR and KOR in this study were 1091.5 and 1212.6 fmol/mg protein separately, and the DOR and KOR cells were mixed at 1:1 ratio of B_{max} for control MDK. The cells coexpressing δ and κ opioid receptors close to a ratio of 1:1 (B_{max} values of δ and κ opioid receptors are 854.2 and 879.3 fmol/mg protein, respectively) were chosen for experimental purposes.

Determination of Protein Concentration and Receptor Ratio. The modified Lowry protein assay was used to determine protein concentration of our samples using bovine serum albumin for establishing a standard curve of absorbance versus micrograms of protein. The sample was determined in triplicate, and the average absorbance value was used to determine the protein concentration of the same sample by comparison with the standard. We used 5 nM [^{3}H]diprenorphine and [^{3}H]naltrindole as tools to determine the ratio of δ and κ opioid receptors. Saturation binding assay was used to determine the maximal binding of [^{3}H]diprenorphine and [^{3}H]naltrindole (B_{max}) to the cells coexpressing δ and κ opioid receptors. The B_{max} values are expressed as femtomoles per milligrams of protein. The B_{max} of [^{3}H]diprenorphine was the total binding to δ and κ opioid receptors, and B_{max} of [^{3}H]naltrindole represented the binding to δ opioid receptors. The difference between the B_{max} of [^{3}H]di-

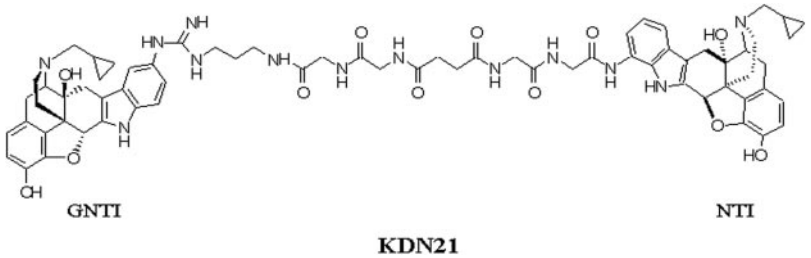

Fig. 1. Stucture of the bivalent ligand KDN21. KDN21 is composed of a δ antagonist pharmacophore, naltrindole (Bhushan et al., 2004), and a κ antagonist pharmacophore, guanidinonaltrindole (Bhushan et al., 2004), linked through a spacer of 21 atoms. The spacer is composed of oligoglycyl units attached to a succinyl core.

prenorphine and the B_{max} of [^{3}H]naltrindole represented the binding to κ opioid receptors.

Radioligand Binding Assay. Cells were grown to approximately 90% confluence in 100-mm dishes, collected using ice-cold trypsin/EDTA, and centrifuged at 1000*g* for 5 min. The cell pellets were rinsed once with ice-cold phosphate-buffered saline, pH 7.4, and centrifuged at 1000*g* for 5 min again. The cells were resuspended in binding buffer (25 mM HEPES, 120 mM NaCl, 1.5 mM $CaCl_2$, 5 mM KCl, and 1.5 mM MgCl2, pH 7.4). Saturation binding was conducted on intact whole cells using radioligands to determine receptor density (B_{max}) and binding affinity (K_d) of the ligand. The cells were incubated with increasing concentrations of a radioligand at 25°C for 2 h in a total volume of 500-μl reaction system. Each concentration was performed in duplicate, and nonspecific binding was defined as the binding of the radioligand not displaced by 10 μM naloxone. The IC_{50} values for tested compounds were determined by competition binding assay in which whole cells were incubated at 25°C for 2 h with an indicated radioligand and nine different concentrations (10^{-14} to 10^{-6} M) of the compounds in a final volume of 500 μl. The concentration of the radioligand used in the competition assay was approximately equivalent to its K_d value, and nonspecific binding was determined as in the presence of 10 μM naloxone. The experiments were repeated three times in duplicate for each assay. Bound radioligand was separated from the free by rapid filtration through Brandel 48-well harvester using Whatman GF/C glass fiber filter paper (Whatman, Clifton, NJ), presoaked in a 0.25% solution of polyethyleneimine in water. The filters with trapped cells were rinsed three times with 4.0 ml of 25 mM HEPES buffer, pH 7.4, precooled to 4°C and placed in scintillation vials with 4.0 ml of Econo-Safe scintillation cocktail and counted in an liquid scintillation counter (LS3801; Beckman Coulter, Fullerton, CA) for 1 min/sample.

MAPK Activity Assay. Cells were grown to approximately 90% confluence in 100-mm dishes, incubated for 6 h, and then incubated with the compounds at 25°C for 10 min. The use of serum-free medium has been shown to reduce the basal level of MAPK activation by inhibiting enzyme phosphorylation (Polakiewicz et al., 1998). Cells were collected using ice-cold trypsin/EDTA and centrifuged at 1000*g* for 5 min. The cell pellets were rinsed once with ice-cold phosphate-buffered saline and centrifuged at 1000*g* for 5 min again. Cell lysates were prepared by the addition of appropriate volume of ice-cold cell lysis buffer (50 mM Tris, 300 mM NaCl, 1% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, and 1 mM $CaCl_2$, pH 7.4) containing 1× protease inhibitor cocktail. The lysates were centrifuged in a microfuge at 12,000*g* for 10 min at 4°C. The supernatants were analyzed for protein concentration using the Modified Lowry Protein Assay and then were prepared for SDS-polyacrylamide gel electrophoresis by boiling them in the sample buffer (125 mM Tris, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.05% bromphenol blue, pH 6.8). Proteins (~10 mg/lane) were separated by SDS-polyacrylamide gel electrophoresis (10% acrylamide running gel and 4% acrylamide stacking gel) and electroblotted onto PVDF membrane (0.45 μm). The PVDF membrane was rinsed three times with TTBS (25 mM Tris, 150 mM NaCl, and 0.1% Tween 20, pH 7.6) and blocked overnight (~12 h) at 4°C in TTBS with 5% nonfat dry milk. Then the PVDF membrane was incubated at 25°C for 2 h with rabbit polyclonal antibodies against phosphorylated MAPK (1:5000 dilution in blocking solution), and after being washed five times for 5 min each time, they were incubated at 25°C for 1 h with donkey anti-rabbit secondary antibodies conjugated to alkaline phosphatase (1:5000). Immunoreacted proteins were visualized using an alkaline phosphatase-sensitive enhanced cyan fluorescent substrate at 25°C for 10 min.

Data Analysis and Statistical Methods. Saturation and competition radioligand binding curves were generated through KaleidaGraph 3.1 (Synergy Software, Reading, PA). The B_{max} and K_d values of the radioligand were determined by Scatchard plotting analysis, in which the *x*-axis is specific binding and the *y*-axis is specific binding divided by free radioligand concentration. IC_{50} values were determined from displacement curves using KaleidaGraph 3.1, and the K_i values were calculated according to the Cheng-Prusoff equation $K_i = IC_{50}/1 + [L]/K_d$ (Cheng and Prusoff, 1973). Images of immunoreactive bands were captured on a Storm PhosphorImager system from GE Healthcare, and the activity of the MAPKs was represented by the gray values of the protein bands on the PVDF membrane. NIH Image software version 1.61 was used to quantify the phosphorylated MAPKs. Statistical data are expressed as mean ± S.E. of the indicated number of observations. Student's *t* test was used to compare the difference between the samples for significance tests.

Results

Binding of KDN Bivalent Ligands to Coexpressed δ and κ Opioid Receptors in HEK293 Cells. In a previous report, we evaluated the KDN series of bivalent ligands using in vitro expression systems. We found that KDN21 possessed the greatest affinity for coexpressed δ and κ opioid receptors when [^{3}H]diprenorphine was used as radioligand (Bhushan et al., 2004). However, because [^{3}H]diprenorphine is a nonselective ligand, we were not able to obtain the information on the binding of KDN21 to individual receptors in the heterodimer. In this study, we used the δ-selective antagonist [^{3}H]natrindole (0.1 nM) and the κ-selective antagonist [^{3}H]nor-BNI (2.0 nM) as radioligands to further evaluate the binding of KDN series members to CDK, MDK, DOR, and KOR in HEK293 cells. It is noteworthy that the binding of [^{3}H]naltrindole and [^{3}H]nor-BNI to coexpressed and singly expressed receptors was similar. The greatly reduced affinity reported (Jordan and Devi, 1999) for the binding of selective ligands to δ-κ heterodimers may have been caused by the use of the nonselective radioligand [^{3}H]diprenorphine. As shown

TABLE 1

Binding (K_i) of KDN bivalent ligands and monovalent controls to coexpressed and singly expressed δ and κ opioid receptors

Competition binding assay was conducted on intact whole cells using 0.1 nM [^{3}H]natrindole or 2.0 nM [^{3}H]nor-BNI as radioligand. Each assay was performed in duplicate, and the data shown here are representative of at least three independent experiments (means ± S.E.).

Ligand	[^{3}H]Naltrindole			[^{3}H]nor-BNI		
	CDK	MDK	DOR	CDK	MDK	DOR
KDN15	3.42 ± 1.06	3.38 ± 1.44	1.92 ± 0.68	3.62 ± 1.41	2.51 ± 1.19	1.82 ± 0.62
KDN20	1.02 ± 0.50	3.23 ± 1.37	2.45 ± 1.09	1.90 ± 0.98	2.62 ± 1.29	2.45 ± 1.19
KDN21	0.06 ± 0.03	1.38 ± 0.57	3.37 ± 1.46	0.16 ± 0.06	4.67 ± 2.06	3.01 ± 1.42
KDN22	2.34 ± 1.08	2.44 ± 1.21	1.23 ± 0.58	4.56 ± 2.04	2.45 ± 1.04	2.18 ± 1.17
KDN23	1.58 ± 0.69	3.62 ± 1.18	7.40 ± 2.20	5.24 ± 2.61	2.34 ± 1.20	2.51 ± 1.36
KDN24	2.04 ± 0.98	7.46 ± 2.57	2.06 ± 0.73	3.88 ± 1.84	2.04 ± 0.93	1.69 ± 0.73
DN21	1.91 ± 0.84	3.29 ± 1.04	1.98 ± 0.52	137.7 ± 54.6	147.6 ± 59.8	203.7 ± 9.98
KN21	197.8 ± 67.9	137.7 ± 55.8	190.1 ± 79.9	4.35 ± 1.74	2.45 ± 1.23	1.58 ± 0.83

in Table 1, when using [^{3}H]natrindole as a label, KDN21 had greater binding affinity for CDK than other KDN members, and relative to MDK and DOR, their K_i values differed by more than 1 order of magnitude. KDN21 also bound more avidly to CDK than did other members and relative to MDK and KOR when [^{3}H]nor-BNI was used as a label. Other bivalent ligands in this series had similar binding affinity for CDK, MDK, DOR, or KOR. The monovalent δ antagonist DN21 and monovalent κ antagonist KN21 exhibited poor ability to displace the binding of [^{3}H]nor-BNI and [^{3}H]natrindole, respectively.

Binding of Naltrindole and nor-BNI to Coexpressed δ and κ Opioid Receptors in HEK293 Cells. Although growing evidence has supported the dimerization/oligomerization of coexpressed opioid receptors, little is known about the roles that dimerization/oligomerization play in the regulation of opioid receptors. We have used the δ-selective antagonist [^{3}H]naltrindole and κ-selective antagonist [^{3}H]nor-BNI to evaluate allosteric effect on the binding of these radioligands to HEK293 cells containing coexpressed δ and κ opioid receptors. We first evaluated their direct binding to CDK. Both [^{3}H]naltrindole and [^{3}H]nor-BNI showed good

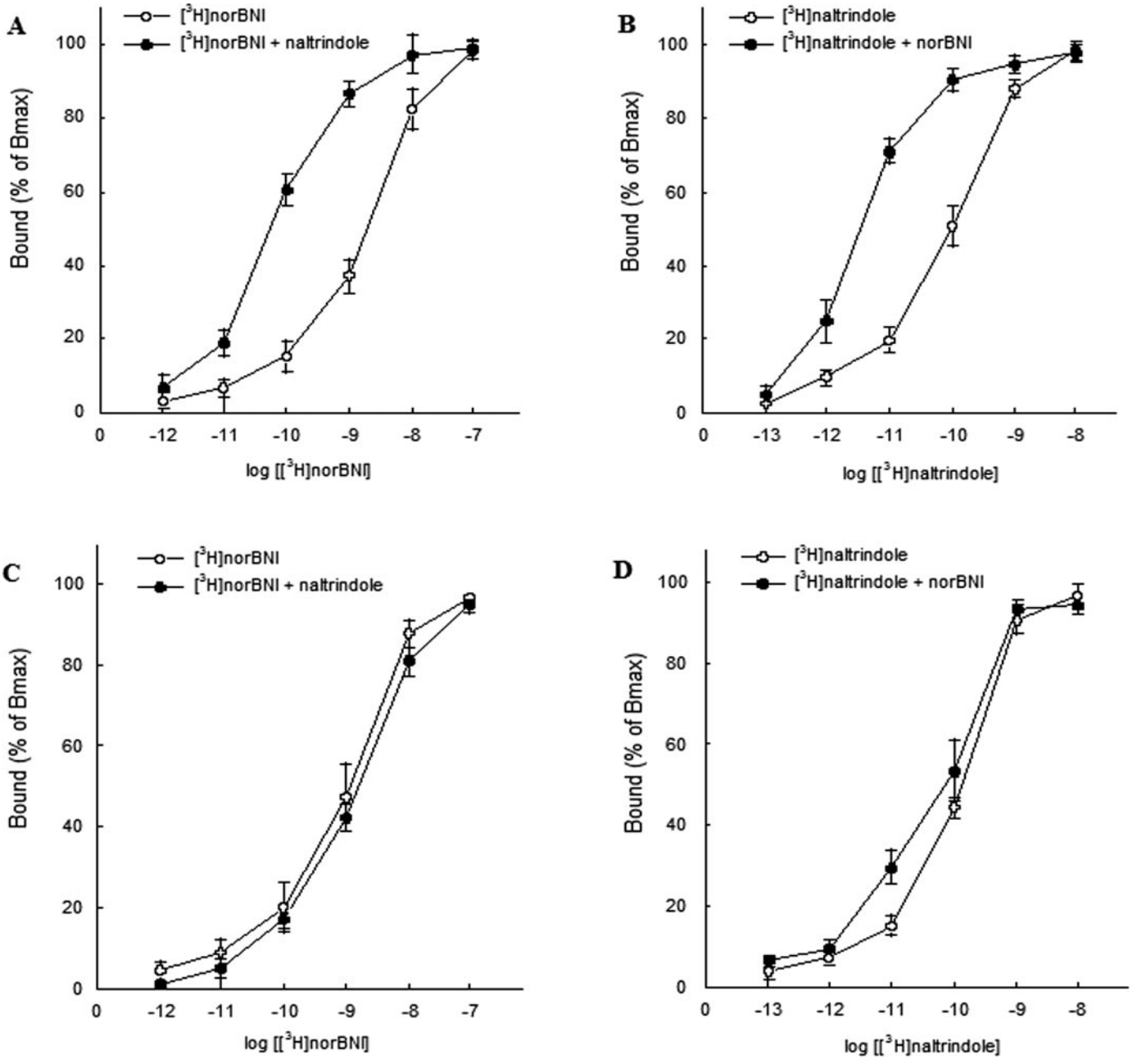

Fig. 2. Binding of naltrindole and nor-BNI to δ and κ opioid receptors coexpressed in HEK293 cells. The binding assay was conducted on intact whole cells using [^{3}H]nor-BNI and [^{3}H]naltrindole as radioligand. Each assay was performed in duplicate, and the data shown are representative of three independent experiments (means ± S.E.). The concentration of cold naltrindole and cold nor-BNI was 10 nM. The cold naltrindole facilitated the binding of [^{3}H]nor-BNI to κ opioid receptors in CDK (A). The binding affinity of [^{3}H]nor-BNI in the presence of naltrindole (●) increases significantly relative to its affinity in the absence of naltrindole (○). Likewise, the cold nor-BNI facilitates the binding of [^{3}H] naltrindole to δ opioid receptors in CDK (B). The binding affinity of [^{3}H]naltrindole in the presence of nor-BNI (●) increases significantly relative to its affinity in the absence of nor-BNI (○). In MDK cells, no such facilitation change occurred (C and D).

affinity for that (Fig. 2, A and B). Their K_d values were 0.1 ± 0.04 and 2.0 ± 0.88 nM, respectively, which were very close to their K_d values for singly expressed δ or κ opioid receptors DOR or KOR (K_d value of [^{3}H]naltrindole for DOR was 0.13 ± 0.05 nM; the K_d value of [^{3}H]nor-BNI for KOR was 1.56 ± 0.72 nM). This suggested that the dimerized δ and κ opioid receptors retained binding to their own selective ligands. To evaluate possible allosteric interaction between δ and κ opioid receptors, we have further investigated the effects of nor-BNI (10 nM) on the binding of [^{3}H]naltrindole and naltrindole (10 nM) on the binding of [^{3}H]nor-BNI to the coexpressed and mixed δ and κ opioid receptors (CDK and MDK) in HEK293 cells. In CDK cells, naltrindole was found to increase the binding affinity of [^{3}H]nor-BNI by 36-fold (Fig. 2A); similarly, nor-BNI increased the binding affinity of [^{3}H]naltrindole by 27-fold (Fig. 2B). It is significant that, in MDK cells, no such facilitation change occurred (Fig. 2, C and D).

Effects of δ- and κ-Selective Ligands on the Binding of KDN21 to Coexpressed δ and κ Opioid Receptors in HEK293 Cells. From the above binding data and our previous studies (Bhushan et al., 2004), in the KDN series, KDN21 is an optimal candidate to bridge δ and κ opioid receptors in vivo and in vitro. In CDK cells, we have found that δ_1, δ_2, κ_1, and κ_2 pharmacological phenotypes coexist on the basis of the activation of ERK1/2 of MAPKs by different selective agonists (Table 2). To determine the selectivity of KDN 21 for such phenotypic receptors, we used selective δ and κ opioid ligands as tools to evaluate their effects on the binding of KDN21 to CDK. When using 0.1 nM [^{3}H]naltrindole as a radioligand in the presence of κ_2 agonist bremazocine (100 nM), the binding curve of KDN21 to CDK was shifted to the right (Fig. 3A), and its K_i value increased 25-fold compared with that in the absence of bremazocine, whereas κ_1 agonist U69593 (100 nM) had little influence. When using 2 nM [^{3}H]nor-BNI in the presence of δ_1 antagonist BNTX (100 nM), the binding curve of KDN21 to CDK was also shifted to the right (Fig. 3B). Its K_i value was 18-fold higher than that in the absence of BNTX. The δ_2 antagonist NTB (100 nM) had little influence in this case. In both cases, the binding of KDN21 to MDK was not changed by δ- or κ-selective ligand (Fig. 3, C and D). BNTX and bremazocine inhibited the binding of KDN21 to CDK cells, but NTB and U69593 did not, which suggests that the bridged receptors are δ_1 and κ_2 phenotypic receptors and provides additional support to our reported intrathecal studies (Bhushan et al., 2004), which revealed that KDN21 selectively antagonized the antinociception induced by administration of DPDPE and bremazocine.

TABLE 2
Effect of KDN21 on the activation of ERK1/2 induced by δ and κ selective agonists

HEK293 cells coexpressing δ and κ opioid receptors were grown to approximately 95% confluence and exposed to serum-free media for 6 h before the addition of the indicated opioid agonist and then incubated with 100 nM DPDPE, deltorphin II, U69593, or bremazocine in the presence or absence of KDN21 (100 nM) at 37°C for 10 min. The cells were extensively washed and prepared for MAPK extraction as described under *Materials and Methods*. Protein (10 μg) was separated via SDS-polyacrylamide gel electrophoresis, followed by immunoblotting using the antibody raised against phosphorylated MAPK. NIH Image software, ver. 1.61, was used to quantify phosphorylated MAPK level. The level of phosphorylated MAPK in the absence of ligand treatment is taken as control and defined as 100%. The data are means ± S.E. of three independent experiments.

Ligand	ERK1/2 Activity
	% of base
DPDPE	304.4 ± 15.4
DPDPE + KDN21	131.0 ± 5.4*
Deltorphin II	229.0 ± 10.1
Deltorphin II + KDN21	220.2 ± 13.4
U69593	255.7 ± 14.0
U69593 + KDN21	253.7 ± 12.7
Bremazocine	278.0 ± 11.2
Bremazocine + KDN21	119.9 ± 6.9*

* Significantly different from the MAPK level in the absence of KDN21 ($p < 0.01$).

Effect of KDN 21 on Phosphorylation of MAPKs Activated by δ- or κ-Selective Agonists. Many GPCRs, including all three opioid receptors μ, δ, and κ, have been reported to activate the MAPKs signaling cascade (Fukuda et al., 1996; Li and Chang, 1996). MAPK activation by GPCRs allows for plasma membrane receptor systems to influence diverse cellular processes, ranging from the regulation of neuronal survival to cell differentiation and gene expression (Gutkind, 1998). Our binding data and intrathecal studies have indicated that KDN21 selectively targets the δ_1 and κ_2 phenotypic opioid receptors. To obtain direct evidence that KDN21 regulates the function of δ_1 and κ_2 opioid phenotypes, we used different δ- and κ-selective agonists to activate the phosphorylation of ERK1/2 and then observed the antagonism of KDN21 upon such activation. In CDK cells, DPDPE, deltorphin II, U69593, and bremazocine could stimulate the phosphorylation of ERK1/2 (Table 2). Compared with the control, they could raise the phosphorylation level of ERK1/2 2- to 3-fold. 100 nM KDN21 could inhibit the activation of ERK1/2 induced by 100 nM DPDPE or 100 nM bremazocine, and the magnitude of inhibition was approximately 2.3-fold ($p < 0.01$). However, no significant inhibition on the activation induced by 100 nM deltorphin II and 100 nM U69593 was observed. These results give direct evidence for the regulation of KDN21 on the function of δ_1 and κ_2 opioid phenotypes.

Discussion and Conclusion

An important issue in opioid research concerns the greater number of putative receptor subtypes than those revealed through cloning. Several reports implicating the existence of δ-κ heterodimeric opioid receptors in vitro and in vivo and have suggested that the putative δ_1 and κ_2 opioid receptor subtypes are, in fact, phenotypes (Portoghese and Lunzer, 2003; Bhushan et al., 2004). δ-κ Opioid receptor heterodimers seemed to display molecular recognition properties that differ from those of their homomeric counterparts (Jordan and Devi, 1999; Bhushan et al., 2004; Daniels et al., 2005). Most recently, this difference has raised the possibility of developing ligands that activate δ-κ heterodimers.

In the present study, we used a bivalent ligand, KDN21, that contains δ and κ antagonist pharmacophores linked through a 21-atom spacer as a tool to investigate the properties of heterodimers of δ and κ opioid receptors in HEK293 cells. In this regard, KDN21 has been reported to have a spacer of optimal distance for bridging δ and κ opioid receptors organized as heterodimers (Bhushan et al., 2004). Although KDN21 possessed the highest affinity for coexpressed δ and κ opioid receptors in HEK293 cells and its in vivo pharmacological selectivity corresponded to the δ_1 and κ_2 phenotypes, the phenotypic receptors were not identified in binding studies because nonselective [^{3}H]diprenorphine was

used as a radioligand. Therefore, in this study, we used selective radioligands to investigate the binding selectivity. With the δ antagonist [^{3}H]naltrindole or the κ antagonist [^{3}H]nor-BNI, coexpressed δ and κ opioid receptors retained good binding selectivity for these selective ligands (Table 1). Using either radioligand, KDN21 showed much higher binding affinity than other members of the series for HEK293 cells coexpressed with both δ and κ receptors (CDK). This provides more direct evidence for the optimal bridging of δ and κ opioid receptors by KDN21.

Pharmacological studies have suggested cooperative interaction between δ and κ opioid receptors, and allosteric coupling has been proposed on the basis of the finding that the κ-selective antagonist nor-BNI antagonizes the δ_1 agonist DPDPE in the spinal cord (Portoghese and Lunzer, 2003). A similar type of antagonism has been reported in the porcine ileum preparation (Poonyachoti et al., 2001). In the both cases, the presence of δ and κ receptors in the same cell has provided support for antagonism being mediated via allosteric heterodimers rather than through cellular circuitry. In the present study, the enhanced affinity of [^{3}H]naltrindole in the presence of nor-BNI suggests that the δ and κ opioid receptors organized as heterodimers function cooperatively (Fig. 2A). That this was a reciprocal effect was demonstrated

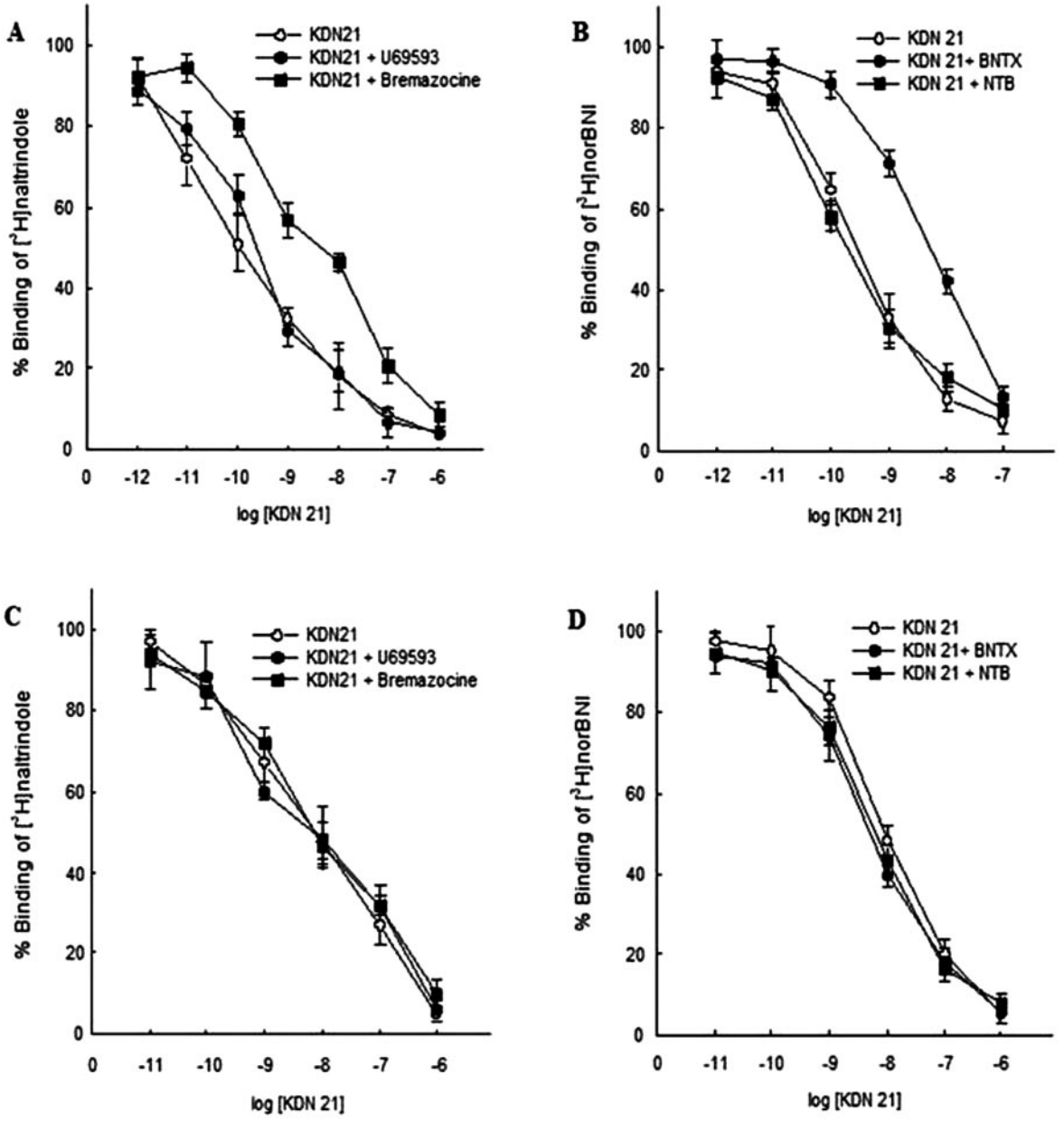

Fig. 3. Effects of δ- and κ-selective ligands on the binding of KDN21 to the CDK. The binding assay was conducted on intact whole cells using [^{3}H]naltrindole or [^{3}H]nor-BNI as radioligand. Each assay was performed in duplicate, and the data shown here are representative of three independent experiments (means ± S.E.). The concentrations of [^{3}H]naltrindole and [^{3}H]nor-BNI were 0.1 and 2.0 nM, respectively, and the concentration of U69593, bremazocine, BNTX, and NTB was 100 nM. When using [^{3}H]naltrindole as a label, bremazocine significantly decreased the binding affinity of KDN21 for CDK, but U695593 showed little influence (A); when [^{3}H]nor-BNI was used as a label, BNTX significantly inhibited the biding of KDN21 to CDK, but NTB showed no such inhibition (B). In the both cases, the binding of KDN21 to MDK were not changed by δ- or κ-selective ligand (C and D).

by the increased affinity of [^{3}H]nor-BNI in the presence of naltrindole (Fig. 2B). These results provide more direct evidence that δ and k opioid receptors function cooperatively as heterodimers. According to the conceptual model illustrated in Fig. 4, δ-κ opioid receptor heterodimers exist either in the agonist or antagonist state, but not in the mixed state. Interaction of a selective antagonist will therefore transform both δ and κ receptor subunits to the antagonist state. Thus, a δ antagonist can antagonize a κ agonist and a κ antagonist can antagonize a δ agonist, both allosterically. The allosteric nature of such coupling has far-reaching implications because antagonism of an agonist by a selective antagonist does not necessarily reveal the receptor that mediates the agonist effect. Given the above results, it seems likely that δ and κ opioid receptor subunits in the heterodimer also facilitate the binding of KDN21 when the spacer permits optimal bridging of the pharmacophores. The binding data for the KDN series (Table 1) are consistent with this view if it is assumed that bridging of KDN21 to the opioid-recognition sites on the heterodimer occurs in discrete steps involving univalent binding followed by the binding of the second pharmacophore (Fig. 5). KDN21 most likely possesses greatly increased affinity relative to other bivalent and monovalent ligands in the series through a combination of positive allosterism and high local concentration of one of the free pharmacophores in the vicinity of the vacant neighboring receptor in the heterodimer. The lower affinity of KDN21, in the presence of κ_2-selective agonist bremazocine or δ_1-selective antagonist BNTX (Fig. 3), is possibly attributable to the competition of them with KDN21 for one of the two binding sites in the heterodimer. Such competition could change the binding of KDN21 from a bivalent state to a univalent state and therefore lead to a lower affinity of KDN21.

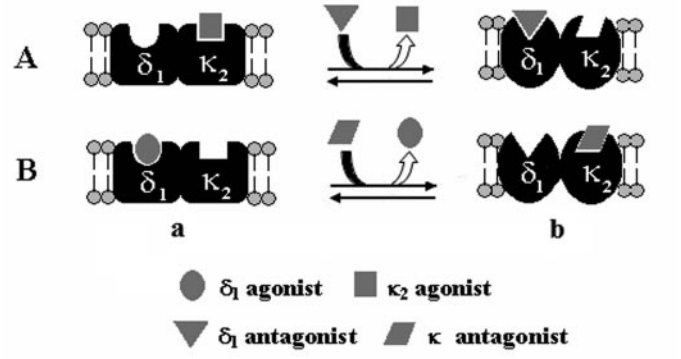

Fig. 4. A conceptional model of the antagonism of a δ_1 or a κ_2 agonist by a κ or a δ_1 antagonist at a δ-κ opioid receptor heterodimer. The change from an agonist state (a) to an antagonist state (b) by the binding of a δ_1 (A) or a κ_2 selective antagonist (B) is accompanied by a conformational change that reduces the affinity of the bound κ_2 or δ_1 agonist.

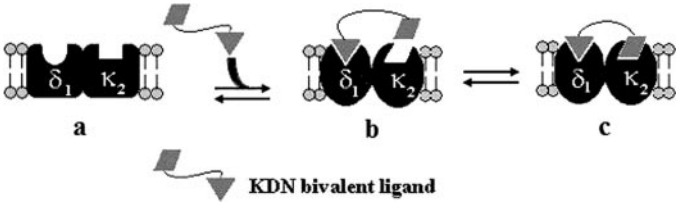

Fig. 5. A conceptual model of the interaction of KDN21 with a δ-κ opioid receptor heterodimer. The δ-κ opioid receptor heterodimer (a) is bound univalently by KDN21 (b). This is accompanied by a conformational change of both receptor subunits that facilitates binding of the second pharmacophore (c). Increased binding of KDN21 to δ-κ opioid receptor heterodimer is also probably caused by the proximity of the unbound pharmacophore to the vacant receptor (b). This model assumes that both subunits are either in the agonist state (a) or antagonist state (b and c).

Intrathecal studies in mice have revealed that KDN21 displays δ_1 and κ_2 selectivity. This was derived from the antagonism of the δ_1 agonist DPDPE and the κ_2 agonist bremazocine. In this regard, the δ_2 agonist deltorphin II and the κ_1 agonist U69593 were only weakly antagonized by KDN21. When taken together with the reports that nor-BNI antagonizes both DPDPE and bremazocine, we concluded that KDN21 interacts selectively with allosteric heterodimeric δ-κ opioid receptors whose phenotypes correspond to δ_1 and κ_2, respectively. To determine whether KDN21 effects the same phenotypic opioid receptors in CDK HEK293 cells, we investigated the antagonism of selective agonist-stimulated phosphorylation of MAPKs (ERK1/2). It is significant that similar antagonist selectivity was observed, in that KDN21 antagonized δ_1-and κ_2-selective agonists in both HEK293 cells and in mice upon intrathecal administration. No significant antagonism of the δ_2 and κ_1 agonists was observed. These results have provided support for the existence δ_1 and κ_2 phenotypic opioid receptors in HEK293 cells. Moreover, the qualitatively similar antagonist selectivity of KDN21 in CDK HEK293 cells lends additional support to the study that KDN21 selectively interacts with δ-κ opioid receptor heterodimers in the mouse spinal cord.

In conclusion, our studies with the bivalent ligand KDN21 provide new insights into the interaction of ligands with δ-κ opioid receptor heterodimers that recognize δ_1- and κ_2-selective ligands. These phenotypic receptors are allosterically coupled and recognize agonists such as DPDPE and bremazocine. The cooperative nature of these heterodimers is manifested by the reported antagonism of a δ_1 agonist (DPDPE) by a κ antagonist (nor-BNI) or the antagonism of a κ_2 agonist (bremazocine) by a δ_1 antagonist (BNTX). In view of these findings, the assignment of a receptor type on the basis of the selectivity of a standard selective opioid antagonist can be problematic. Finally, KDN21 should be a useful tool for identifying δ-κ opioid receptor heterodimers both in vitro and in vivo.

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