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The in vitro pharmacological characterization of naloxone benzoylhydrazone

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Abstract

On the basis of its in vivo activity and binding affinity, naloxone benzoylhydrazone has been characterized as a κ_3-opioid receptor agonist and a μ-opioid receptor antagonist. This paper continues its pharmacological characterization with the help of isolated tissue preparations. Naloxone benzoylhydrazone was found to have partial agonist activity in the guinea pig ileum longitudinal muscle/myenteric plexus preparation. As an antagonist, naloxone benzoylhydrazone is similar to naloxone, with pA_2 values of 8.8, 7.8, and 7.8 for μ-, δ-, and κ_1-opioid receptors, respectively. Its agonist activity in the guinea pig ileum preparation was not influenced by β-funaltrexamine treatment but was reversed by the selective κ-opioid receptor antagonist nor-binaltorphimine and by the irreversible κ_1-opioid receptor blocker UPHIT (1*S*,2*S*)-*trans*-2-isothiocyanato-4,5-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl] benzeneacetamide. The presence of κ_3-opioid receptors could not be demonstrated by [^{3}H]naloxone benzoylhydrazone binding in the guinea pig ileum longitudinal muscle/myenteric plexus preparation. From these studies it is concluded that the partial agonist activity of naloxone benzoylhydrazone in this bioassay is probably due to the activation of the κ_1-opioid receptors.

Keywords: Naloxone benzoylhydrazone; Ileum, guinea pig; κ-Opioid receptor; Receptor binding

1. Introduction

Earlier reports (Clark et al., 1989; Gistrak et al., 1989; Price et al., 1989; Paul et al., 1990) described the binding and in vivo pharmacological profile of the opiate agonist/antagonist naloxone benzoylhydrazone (6-desoxy-6-benzoylhydrazido-*N*-allyl-14-hydroxy-dihydronormophinone). In vivo experiments up to a mg/kg using naloxone benzoylhydrazone in mice showed the compound to be a μ-opioid receptor antagonist. Higher doses, however, caused analgesia, which could be reversed by the κ-opioid antagonist WIN 44,441 ([3(2-α,6-α,11*S*)-(−)-1-cyclopentyl-5-(1,2,3,4,5,6-hexahydro-8-hydroxy-3,6,11-trimethyl-2,6-methano-3-benzazocin-11-yl)pentanone) and less effectively by naloxone. Furthermore, there was no cross-tolerance between naloxone benzoylhydrazone and either morphine or the κ_1-opioid receptor selective agent U 50,488 (*trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide), but cross-tolerance was demonstrated with the agonist/antagonist nalorphine. This in vivo profile was reminiscent of the κ-opioid receptor described by Martin et al. (1976), and it was suggested that analgesia was effected through a selective κ_3-opioid receptor mechanism.

Clark et al. (1989) demonstrated [^{3}H]naloxone benzoylhydrazone binding to a site with high density in calf striatum (B_{max} 14.6 ± 0.69 fmol/mg). This κ_3-opioid receptor has high affinity for naloxone benzoylhydrazone (K_d = 0.74 ± 0.04 nM) and other agonist/antagonist compounds, but it also has relatively high affinity for putative μ-selective compounds such as DAMGO ([D-Ala^2,(Me)Phe^4,Gly-ol^5]enkephalin) (K_i = approximately 10 nM). This site is different from κ_1, which has been defined as having high affinity for U

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50,488, and from κ_2, which has been shown to have low affinity for peptides such as DAMGO (Lahti et al., 1985; Price et al., 1989; Zukin et al., 1988). Of these three sites, only κ_1 has been well defined, progress that was possible because of the availability of the selective agonists U 50,488 and U 69,593 ([5α,7α,8β]-(+)-*N*-methyl-*N*-[7-(*l*-pyrrolidinyl)-1-oxaspiro-(4,5)-dec-8-yl]) and antagonist nor-binaltorphimine. Although there are high affinity agonists for the κ_3 site (such as naloxone benzoylhydrazone), no selective agonists or antagonists are known, and no in vitro activity has been measured. Our aim was to further characterize naloxone benzoylhydrazone's activity with the help of in vitro bioassays and, if possible, to determine whether κ_3-opioid receptors are present in the longitudinal muscle/myenteric plexus of the guinea pig ileum.

2. Materials and methods

2.1. Functional assays

Longitudinal muscle / myenteric plexus of guinea pig ileum (LMMP)

Male Hartley guinea pigs weighing 350–400 g were decapitated and the small intestine removed. The longitudinal muscle with the myenteric plexus attached was gently separated from the underlying circular muscle by the method of Paton and Vizi (1969). The muscle strips were mounted in an 8-ml, water-jacketed organ bath containing Krebs-bicarbonate solution of the following composition (in mM): NaCl 118, $CaCl_2$ 2.5, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, and glucose 11.5. The tissues were kept at 37° C and bubbled with 5% CO_2 in oxygen. An initial tension of 1.0 g was applied to the strips. The tissues were electrically stimulated for 60 min before the start of the experiments. Field electrical stimulation was applied, delivered through platinum wire electrodes positioned at the top and bottom of the organ bath and kept a fixed distance (3.5 cm) apart. The parameters of rectangular stimuli were as follows: supramaximal voltage, 1 ms impulse duration at 0.1 Hz.

Electrically stimulated mouse vas deferens (MVD)

Swiss-Webster mice weighing 30–35 g were used. The vasa deferentia were prepared according to the method of Hughes et al. (1975) and bathed at 31° C in Mg^{2+}-free Krebs solution, bubbled with a mixture of oxygen and carbon dioxide (95 : 5). An initial tension of 150–200 mg was applied. The experiments were carried out in an organ bath of 8 ml capacity. The parameters of field stimulation were slightly modified from those in the original description (Ronai et al., 1977). Paired shocks with a 100-ms delay between supramaximal rectangular pulses of 1 ms duration, delivered at a rate of 0.1 Hz, were used.

In both assays, the electrically induced contractions were recorded using an isometric transducer (Metrigram) and either a Grass 7D multichannel polygraph or a Macintosh SE/30 computer connected to a MacLab system and a Transbridge amplifier. For the electrical stimulation, a Grass S-88 electrostimulator was used.

Agonist / antagonist determinations

The agonist potencies of compounds were determined from concentration-response curves and characterized by the IC_{50} values. Agonists were present for 3–6 min or until maximal inhibitory effect was produced. Cumulative concentration-response curves were made for U 69,593 and naloxone benzoylhydrazone. The percent inhibition of the stimulation-induced contraction produced by each agonist was plotted against the log agonist concentration. IC_{50} is defined as the concentration of the agonist that produces 50% inhibition of the contraction.

Antagonists were present for at least 30 min. Antagonist activities were calculated for each single tissue from full concentration-response curves before and after addition of a single antagonist concentration. The dose ratio (DR) is the shift of the agonist concentration-response curve in the presence of the antagonist. At least three different concentrations were used, and only one antagonist concentration was tested on each tissue. pA_2 values were determined from Schild plots (Arunlakshana and Schild, 1959). Each individual point was plotted, and pA_2 was determined using a statistical least-squares regression analysis program package (R. Barlow, UK).

2.2. Receptor binding studies

Receptor binding studies in the brain

For studies of binding to whole guinea pig brain, brains were quickly removed from male Hartley guinea pigs and homogenized using a Polytron homogenizer (Brinkman) in 50 mM Tris, pH 7.7. The homogenate was centrifuged at 40 000 × *g* for 15 min, and the pellet was rehomogenized in Tris and centrifuged once more. The final pellet was resuspended at a concentration of 6.6 mg tissue/ml Tris.

For the binding assays, 1.8 ml membrane preparation was incubated with 0.2 nM [^{3}H]naloxone benzoylhydrazone and the appropriate test compound in a total volume of 2.0 ml for 1 h at 25° C. Also present in the incubation medium was 5 mM EDTA to reduce naloxone benzoylhydrazone binding to the μ-opioid receptors and, in the case of peptides, 50 μg/ml bacitracin to prevent proteolysis. U 69,593, 100 nM, was used to block binding to κ_1-opioid receptors. The 50

μg/ml bacitracin was found to totally inhibit degradation of dynorphin during the course of the incubation without affecting [^{3}H]naloxone benzoylhydrazone binding. For most of the competition experiments, 14 concentrations of the competing ligand were used. At the end of the incubation, the samples were filtered over Whatman GF/B filters by using a Brandel cell harvester. Nonspecific binding was determined using 10 μM naloxone benzoylhydrazone.

[^{3}H]Bremazocine (1.1 nM) binding to the κ_2-opioid receptors in brain membranes was conducted as described previously (Webster et al., 1993) with 100 nM DAMGO, 100 nM DPDPE ([D-Pen^2,D-Pen^5]enkephalin), and 100 nM U 69,593 used to block binding to μ-, δ-, and κ_1-opioid receptors, respectively.

Receptor binding studies in the guinea pig ileum

For studies of binding to guinea pig ileum, the method of Webster et al. (1993) was used. The binding assays were conducted in a total volume of 0.5 ml, with the incubation lasting 60 min at 37° C. As with brain membranes, 5 mM EDTA was present to block binding to the μ-opioid receptors and 50 μg/ml bacitracin to inhibit degradation of peptides. At the conclusion of the incubation period, the samples were filtered over Whatman GF/B glass fiber filters by using a Brandel cell harvester. Nonspecific binding was determined using 10 μM naloxone benzoylhydrazone.

The internationally accepted principles of animal care and use were adhered to in these studies.

Data analysis

All of the experiments were initially analyzed using the program ALLFIT (De Lean et al., 1978). ALLFIT derives IC_{50} values and slope factors based upon the competition experiments. Saturation binding experiments were analyzed using the program LIGAND (Munson and Rodbard, 1980). Both programs were the kind gift of Dr. David Rodbard (of the U.S. National Institutes of Health).

2.3. Materials

[^{3}H]Bremazocine (30 Ci/mmol) was obtained from Dupont/New England Nuclear, bremazocine was a gift from Dr. James Woods (University of Michigan), and [^{3}H]naloxone benzoylhydrazone and naloxone benzoylhydrazone were synthesized as described by Luke et al. (1988). β-Funaltrexamine and nor-binaltorphimine dihydrochloride were obtained from Research Biochemicals International, UPHIT (1*S*,2*S*-*trans*-2-iso-thiocyanato-4,5-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzen-acetamide) was synthesized as described by De Costa et al. (1989). CTAP-NH_2 [D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2], DAMGO ([D-Ala^2,*N*-Me-Phe^4,Gly-ol^5]enkephalin), DPDPE ([D-Pen^2,D-Pen^5]enkephalin), dynorphin-(1–13)-OH, naloxone, U 50,488 and U 69,593 were obtained from the National Institute on Drug Abuse.

3. Results

The longitudinal muscle/myenteric plexus of the guinea pig ileum has been shown to have functional μ- and κ- but not δ-opioid receptors (Chavkin et al., 1982; Leslie et al., 1980; Lord et al., 1977). Naloxone benzoylhydrazone was found to be a partial agonist in the longitudinal muscle/myenteric plexus of the guinea pig ileum. The inhibition started at a naloxone benzoylhydrazone concentration of 10 nM and even at 1000 nM was less than 50% (the amount of inhibition varied between tissues), with the curve being extremely shallow (see Fig. 1). First we studied naloxone benzoylhydrazone's antagonist activity at the μ- and κ_1-opioid receptors in the longitudinal muscle/myenteric plexus of the guinea pig ileum by examining inhibition of the effects of the selective agonists DAMGO and U 69,593, respectively.

Because of the partial agonist activity of naloxone benzoylhydrazone in the longitudinal muscle/myenteric plexus of the guinea pig ileum we were concerned about the accuracy of our pA_2 determination in that tissue, so we repeated the pA_2 determination in the mouse vas deferens where naloxone benzoylhydrazone has negligible agonist activity. In this tissue, antagonist activity was examined at μ-, δ-, and κ_1-opioid receptors. The results obtained in the two assay systems (see Table 1) were in very close agreement for the μ- and κ_1-opioid receptors. In addition

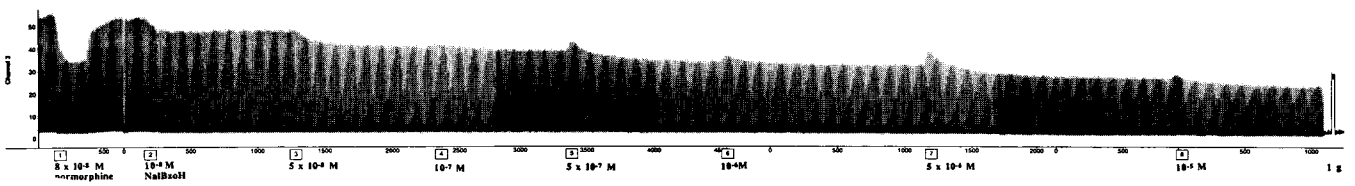

Fig. 1. Cumulative concentration-response curve for naloxone benzoylhydrazone in the longitudinal muscle/myenteric plexus of the guinea pig ileum. Concentrations injected were as follows: 10^{-8}, 5×10^{-8}, 10^{-7}, 5×10^{-7}, 10^{-6}, 5×10^{-6} and 10^{-5} M. Injections were made 15–20 min apart. The tissue sensitivity was checked out by injecting 80 nM normorphine at the end of the 60-min equilibrium time.

Table 1
pA_2 determination for the antagonist effects of naloxone benzoylhydrazone (NalBzoH) at μ-, κ_1-, and δ-opioid receptors in two isolated tissue prepartions

Tissue	Antagonist	pA_2 / *Schild slope*		
		μ	κ	δ
LMMP	NalBzoH	8.81 ± 0.10	7.76 ± 0.03	
		-1.02 ± 0.14	-1.19 ± 0.04	
LMMP	Naloxone	8.51 ± 0.03	7.73 ± 0.01	
		-1.07 ± 0.04	-0.99 ± 0.03	
LMMP	CTAP	7.65 ± 0.01		
		-1.02 ± 0.02		
MVD	NalBzoH	8.82 ± 0.01	7.45 ± 0.04	7.76 ± 0.03
		-0.91 ± 0.02	-1.09 ± 0.08	-0.96 ± 0.05
MVD	Naloxone			7.30 ± 0.10
				-1.05 ± 0.10

pA_2 determinations were made on the longitudinal muscle/myenteric plexus of the guinea pig ileum (LMMP) and mouse vas deferens (MVD) as described in Materials and methods.

the pA_2 values found for naloxone benzoylhydrazone were very similar to that found for naloxone. Naloxone benzoylhydrazone and naloxone are about 10 times as potent against μ ligands as they are against δ and κ ligands.

To determine the nature of the partial agonist activity of naloxone benzoylhydrazone in the longitudinal muscle/myenteric plexus of the guinea pig ileum, selective antagonists were used to inhibit its agonist activity. Both the μ-selective antagonist CTAP ([D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2]) (Kramer et al., 1989) and the κ-selective antagonist nor-binaltorphimine (Takemori et al., 1988) bind with high affinity to the κ_3-opioid receptors (see Table 2). If the agonist activity of naloxone benzoylhydrazone occurred through activation of the κ_3-opioid receptors in the longitudinal muscle/myenteric plexus of the guinea pig ileum, we would expect both antagonists to reverse or block the naloxone benzoylhydrazone-induced inhibition of twitch. However, we found that the naloxone benzoylhydrazone-induced inhibition could be reversed by nor-binaltorphimine but not by CTAP up to concentrations of 1000 nM. The fact that only nor-binaltorphimine was effective as an antagonist suggests that the agonist activity of naloxone benzoylhydrazone occures through κ_1- rather than κ_3-opioid receptors. Further proof for κ_1-opioid receptor involvement was obtained when tissues were pretreated with the irreversible μ-opioid receptor blocker β-funaltrexamine (Ward et al., 1982), 200 nM for 30 min or the guinea pigs were injected with the irreversible κ_1-opioid receptor blocker UPHIT, 20 mg/kg intraperitoneally (De Costa et al., 1989). β-Funaltrexamine-treated tissues showed the same agonist activity for naloxone benzoylhydrazone as they had before the treatment. However, when the guinea pigs were pretreated with UPHIT 48 h before the experiment, the partial agonist activity of naloxone benzoylhydrazone was completely abolished (Fig. 2). These findings strongly suggest a κ_1-opioid receptor involvement in naloxone benzoylhydrazone's partial agonist activity in the longitudinal muscle/myenteric plexus of the guinea pig ileum.

To further examine the longitudinal muscle/myenteric plexus of the guinea pig ileum for κ_3-opioid receptors, binding studies were conducted using [^{3}H]naloxone benzoylhydrazone. [^{3}H]Naloxone benzoylhydrazone affords excellent specific binding to the guinea pig ileum membranes, achieving greater than 90% specific binding. Analysis of self-inhibition experiments (Fig. 3) using the curve-fitting program LIGAND (Munson and Rodbard, 1980) indicated a K_d of 46 ± 15 nM and a B_{max} of 30 ± 8.4 fmol/mg protein for [^{3}H]naloxone benzoylhydrazone. A two-binding-site model was also attempted using LIGAND. Although the mean square error decreased significantly for a two-site model, the error derived for each parameter was greater than 100%, so it was impossible to determine affinities and binding capacities.

Table 2
Comparison of [^{3}H]naloxone benzoylhydrazone (NalBzoH) binding to guinea pig ileum and brain membranes with [^{3}Hbremazocine binding to κ_2-opioid receptors in brain membranes

Cold ligand	[^{3}H]NalBzoH binding in the brain IC_{50} (nM) κ_3	[^{3}H]NalBzoH binding in the ileum IC_{50} (nM)	[^{3}H]Bremazocine binding in the brain IC_{50} (nM) κ_2
Bremazocine	1.1 ± 0.53	46 ± 15	11.2 ± 1.2
CTAP	4.9 ± 2.05	–	> 10000
DAMGO	47.0 ± 6.08	767 ± 329	> 10000
Dynorphin-(1–13)-OH	9.6 ± 5.59	145 ± 101	43.2 ± 20.1
NalBzoH	0.7 ± 0.13	35 ± 18	81.5 ± 26.2
Nor-BNI	25.2 ± 1.6	66.2 ± 37.0	37.5 ± 3.33
U 50,488	233.5 ± 94.0	–	307.5 ± 61.5
U 69,593	1978.5 ± 304	3000 ± 1400	6763 ± 418
UPHIT	1394.5 ± 695	781.5 ± 300	428.0 ± 63.0

Data shown are IC_{50} values ± S.D. derived from at least two experiments conducted in triplicate. [^{3}H]Bremazocine binding to brain membranes was conducted in the presence of 100 nM DAMGO, DPDPE, and U 69,593 to block binding to μ-, δ-, and κ_1-opioid receptors, respectively.

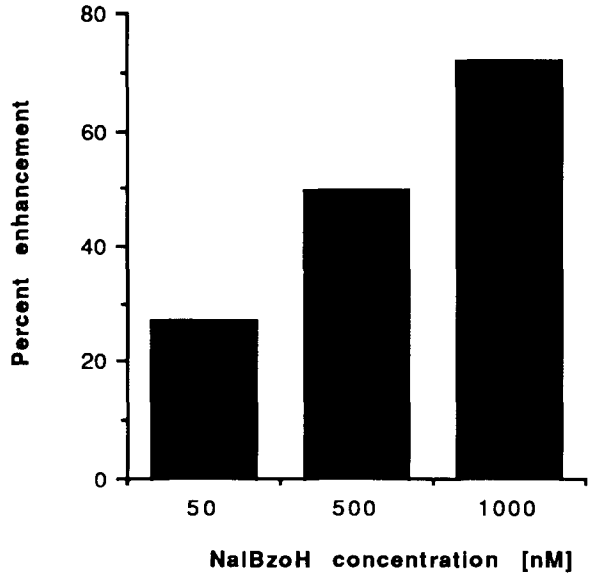

Fig. 2. The graphs represent the percent enhancement produced by naloxone benzoylhydrazone in the UPHIT-treated longitudinal muscle/myenteric plexus of the guinea pig ileum. The graphs represent the average enhancement of two tissues only.

We have previously reported that κ_2-opioid receptors found in the guinea pig ileum closely resemble those found in guinea pig brain membranes (Webster et al., 1993). If we compare the IC_{50} values for a similar set of compounds at the κ_3 site in the brain and [^{3}H]naloxone benzoylhydrazone binding in the guinea pig ileum, we find that the affinities do not correspond well at all (Table 2). In particular, the IC_{50} values of naloxone benzoylhydrazone and DAMGO for inhibition of [^{3}H]naloxone benzoylhydrazone binding in brain membranes were 0.7 and 47 nM, respectively. In guinea pig ileum membranes, the respective IC_{50} values were 35 and 767 nM. Moreover, the low affinities derived for DAMGO and U 69,593 indicate that [^{3}H]naloxone benzoylhydrazone is not binding primarily to μ or κ_1 sites. Fig. 3 shows the inhibition curves from which the data in Table 2 were derived. As seen in this figure, there does appear to be a small, high affinity component of both DAMGO and U 69,593 inhibition. This is consistent with pseudo-Hill coefficients of approximately 0.8 for most of the inhibition curves and suggests that [^{3}H]naloxone benzoylhydrazone does bind to a small extent to μ- and κ_1-opioid receptors that are known to be present in the longitudinal muscle/myenteric plexus of the guinea pig ileum. Although it is not a perfect fit, the majority of the data corresponds more closely to the κ_2-opioid binding reported by us (Webster et al., 1993) and others (Tiberi and Magnan, 1989). These findings suggest that the guinea pig ileum does not have κ_3-opioid receptors similar to those in the guinea pig brain, and that the binding of [^{3}H]naloxone benzoylhydrazone found in the longitudinal muscle/myenteric plexus of the guinea pig ileum may represent binding to κ_2 or other low affinity receptors.

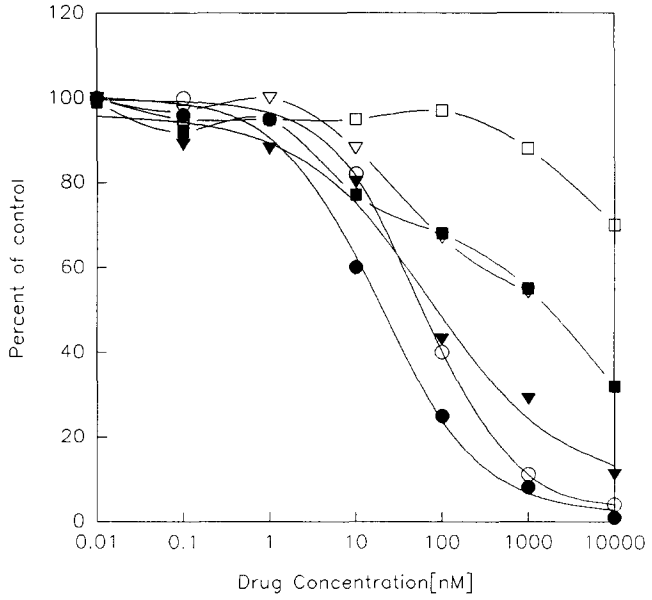

Fig. 3. Inhibition of [^{3}H]naloxone benzoylhydrazone binding to guinea pig ileum membranes by opioid compounds. Binding was conducted as described in Materials and methods. The opioid compounds used were naloxone benzoylhydrazone (●), bremazocine (○), nor-binaltorphimine (▼), DAMGO (▽), U 69,593 (■), and DPDPE (□). Data shown are from single experiments conducted in triplicate. Each compound was tested 2 or 3 times and the results were similar.

4. Discussion

Although naloxone benzoylhydrazone has been characterized in terms of binding and in vivo pharmacological profiles, no quantitative information was available about its antagonist properties, since it is very difficult to determine accurate pA_2 values from in vivo experiments. Our bioassay results showed that naloxone benzoylhydrazone and naloxone are equally active in antagonizing all three opioid receptors, and they are both about 10-fold weaker at the δ and κ_1 sites than at μ sites.

The agonist activity of naloxone benzoylhydrazone is more difficult to characterize. Even in vivo, naloxone benzoylhydrazone has poor agonist activity, acting as an agonist only in certain strains of mice (unpublished observation). Furthermore, cross-tolerance is produced with the agonist/antagonist nalorphine but not with the prototypical μ and κ_1 agonists morphine and U 69,593. This behavior, along with specific [^{3}H]naloxone benzoylhydrazone binding characteristics, led to the hypothesis that analgesia was through a novel receptor type (κ_3).

In the longitudinal muscle/myenteric plexus preparation of the guinea pig ileum, naloxone benzoylhydrazone is clearly a partial agonist, never reaching greater than 50% inhibition of twitch (see Fig. 1). This finding is consistent with the observation by Gistrak et al. (1989) that naloxone benzoylhydrazone produces limited inhibition of gastrointestinal transit in mice, with a ceiling effect. Several experiments were conducted to determine the site mediating this agonist activity. The partial agonist activity of naloxone benzoylhydrazone was reversed by the selective κ antagonist nor-binaltorphimine, in contrast to the analgesic activity of naloxone benzoylhydrazone, which is insensitive to nor-binaltorphimine (Paul et al., 1990). In addition, the agonist activity of naloxone benzoylhydrazone was inhibited by pretreatment with the irreversible κ_1-opioid receptor antagonist UPHIT, which has high affinity for κ_1 but not κ_3 sites (Table 2), but not by the irreversible μ-opioid receptor antagonist β-funaltrexamine. These experiments suggest that the agonist activity of naloxone benzoylhydrazone in the longitudinal muscle/myenteric plexus of the guinea pig ileum is probably mediated by κ_1-opioid receptors. They also give a further indication that the benzeneacetamide structure of UPHIT and U 69,593 does not bind to κ_3-opioid receptors and can be used to selectively block the activity of κ_1-opioid receptors.

The absence of functional receptors in the longitudinal muscle/myenteric plexus of the guinea pig ileum does not mean that the receptor type is not found in that tissue. Both δ- (Leslie et al., 1980) and κ_2-opioid (Webster et al., 1993) receptors can be characterized by binding or other assays in guinea pig ileum. However, δ-opioid receptors (Leslie et al., 1980) do not mediate an inhibition of twitch in this preparation, nor is there evidence that κ_2-opioid receptors mediate a functional response. Binding studies were also conducted in the longitudinal muscle/myenteric plexus of the guinea pig ileum with [^{3}H]naloxone benzoylhydrazone to examine this tissue for the presence of κ_3-opioid receptors. Although [^{3}H]naloxone benzoylhydrazone exhibits considerable specific binding, it does not resemble κ_3-opioid receptor binding in brain membranes. The IC_{50} values determined correspond more closely to [^{3}H]bremazocine binding to κ_2-opioid receptors in both guinea pig ileum and brain membranes. In particular, naloxone benzoylhydrazone has an IC_{50} value of 0.7 nM for κ_3-opioid receptor binding in brain membranes, while the IC_{50} value in guinea pig ileum is approximately 35 nM. This is more similar to an IC_{50} of 81.5 nM for inhibition of [^{3}H]bremazocine binding to κ_2-opioid receptors in brain (Table 2). Furthermore, DAMGO, which has reasonably high affinity for the [^{3}H]naloxone benzoylhydrazone binding site in brain, has quite low affinity ($IC_{50} = 767$ nM) in guinea pig ileum membranes. Finally, the B_{max} value derived from [^{3}H]naloxone benzoylhydrazone binding to the guinea pig ileum is similar to that previously reported for binding of [^{3}H]bremazocine to the same preparation (Webster et al., 1993). However, only K_i values can be directly compared at different receptors. The values we report here are IC_{50} values because the low Hill coefficients suggest the presence of multiple sites, but the curve-fitting program was not able to successfully derive binding affinities at more than a single site. IC_{50} values can best be used to determine rank order of potencies at different receptors. There appear to be differences in rank order between [^{3}H]naloxone benzoylhydrazone binding in guinea pig ileum membranes and κ_3-opioid receptor binding in brain membranes. Therefore the nature of this binding site remains to be determined.

The apparent lack of κ_3-opioid receptors in the guinea pig ileum is consistent with our conclusion from activity studies that the partial agonist activity of naloxone benzoylhydrazone, in this tissue, is due to κ_1-opioid receptor activation.

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References

Arunlakshana, O. and H.O. Schild, 1959, Some quantitative uses of drug antagonists, Br. J. Pharmacol. 14, 48.

Chavkin, C., I.F. James and A. Goldstein, 1982, Dynorphin is a specific endogenous ligand of the κ opioid receptor, Science 215, 413.

Clark, J.A., L. Liu, M. Price, B. Hersh, M. Edelson and G.W. Pasternak, 1989, Kappa opiate receptor multiplicity: evidence for two U50,488-sensitive κ_1 subtypes and a novel κ_3 subtype, J. Pharmacol. Exp. Ther. 251, 461.

De Costa, B.R., L. Band, R.B. Rothman, A.E. Jacobson, V. Bykov, A. Pert and K.C. Rice, 1989, Synthesis of an affinity ligand ('UPHIT') for in vivo acylation of the κ-opioid receptor, FEBS Lett. 249, 178.

DeLean, A., P.J. Munson and D. Rodbard, 1978, Simultaneous analysis of families of sigmoidal dose-response curves: application to bioassay, radioligand assay, and physiological dose-response curves, Am. J. Physiol. 235, E97.

Gistrak, M.A., D. Paul, E.F. Hahn and G.W. Pasternak, 1989, Pharmacological actions of a novel mixed opiate agonist/antagonist: naloxone benzoylhydrazone, J. Pharmacol. Exp. Ther. 251, 469.

Hughes, J., H.W. Kosterlitz and F.M. Leslie, 1975, Effect of morphine on adrenergic transmission in the mouse vas deferens: assessment of agonist and antagonist potencies of narcotic analgesics, Br. J. Pharmacol. 53, 371.

Kramer, T.H., J.E. Shook, W. Kazmierski, E.A. Ayres, W.S. Wire,

V.J. Hruby and T.F. Burks, 1989, Novel peptidic mu opioid antagonists: pharmacologic characterization in vitro and in vivo, J. Pharmacol. Exp. Ther. 249, 544.

Lahti, R.A., M.M. Mickelson, J.M. McCall and P.F. Von Voigtlander, 1985, [^{3}H]U-69593 a highly selective ligand for the opioid κ receptor, Eur. J. Pharmacol. 109, 281.

Leslie, F.M., C. Chavkin and B.M. Cox, 1980, Opioid binding properties of brain and peripheral tissues: evidence for heterogeneity in opioid ligand binding sites, J. Pharmacol. Exp. Ther. 214, 395.

Lord, J.A.H., A.A. Waterfield, J. Hughes and H.W. Kosterlitz, 1977, Endogenous opioid peptides: multiple agonists and receptors, Nature 267, 495.

Luke, C.M., E.F. Hahn, M. Price and G.W. Pasternak, 1988, Irreversible opiate agonists. V. Hydrazone and acylhydrazone derivatives of naltrexone, Life Sci. 43, 1249.

Martin, W.R., C.G. Eades, J.A. Thompson, R.E. Huppler and P.E. Gilbert, 1976, The effects of morphine- and nalorphine-like drugs in the nondependent and morphine-dependent chronic spinal dog, J. Pharmacol. Exp. Ther. 197, 517.

Munson, P.J. and D. Rodbard, 1980, LIGAND: a versatile computerized approach for characterization of ligand-binding systems, Anal. Biochem. 107, 220.

Paton, W.D.M. and E.S. Vizi, 1969, The inhibitory action of noradrenaline and adrenaline on acetylcholine output on guinea-pig longitudinal muscle strip, Br. J. Pharmacol. 35, 10.

Paul, D., J.A. Levison, D.H. Howard, C.G. Pick, E.F. Hahn and G.W. Pasternak, 1990, Naloxone benzoylhydrazone (NalBzoH) analgesia, J. Pharmacol. Exp. Ther. 255, 769.

Price, M., M.A. Gistrak, Y. Itzhak, E.F. Hahn and G.W. Pasternak, 1989, Receptor binding of [^{3}H]naloxone benzoylhydrazone: a reversible κ and slowly dissociable μ opiate, Mol. Pharmacol. 35, 67.

Ronai, A.Z., L. Graf, J.I. Szekely, Zs. Dunai-Kovacs and S. Bajusz, 1977, Differential behaviour of LPH-(61–69)-peptide in different model systems: comparison of the opioid activities of LPH-(61–69)-peptide and its fragments, FEBS Lett. 74, 182.

Takemori, A.E., B.Y. Ho, J.A. Naeseth and P.S. Portoghese, 1988, Nor-binaltorphimine, a highly selective kappa-opioid antagonist in analgesic and receptor binding assays, J. Pharmacol. Exp. Ther. 246, 255.

Tiberi, M. and J. Magnan, 1989, Pharmacological characterization of the binding of [^{3}H]bremazocine in guinea pig brain: evidence for multiplicity of the κ-opioid receptor, Can. J. Physiol. Pharmacol. 67, 1336.

Ward, S.J., P.S. Portoghese and A.E. Takemori, 1982, Pharmacological profiles of β-funaltrexamine (β-FNA) and β-chlornaltrexamine (β-CNA) on the mouse vas deferens preparation, Eur. J. Pharmacol. 80, 377.

Webster, J.L., W.E. Polgar, S.R. Brandt, I.P. Berzetei-Gurske and L. Toll, 1993, Comparison of κ_2-opioid receptors in guinea pig brain and guinea pig ileum membranes, Eur. J. Pharmacol. 231, 251.

Zukin, R.S., M. Eghbali, D. Olive, E.M. Unterwald and A. Temple, 1988, Characterization and visualization of rat and guinea pig brain κ opioid receptors: evidence for κ_1 and κ_2 opioid receptors, Proc. Natl. Acad. Sci. USA 85, 4061.