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Report

A Naloxone–Steroid Hybrid Azine with Selective and Long-Acting Opioid Antagonism at Delta Receptors *In Vitro*

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The interaction of naloxone estrone azine (N-EH) with various opioid receptor types was studied *in vitro*. Its potency as an antagonist of opioid effects was compared to that of naloxone on the electrically evoked contractions of mouse vas deferens (Mvd) and guinea pig ileum myenteric plexus longitudinal muscle (Gpi) preparations. N-EH was found to be 9-fold more potent than naloxone in antagonizing the effects of D-Ala^2-Leu^5-enkephalin in the Mvd and 22-fold less potent in antagonizing normorphine in the Gpi. In the Mvd, the recovery half-time for N-EH was longer than 1000 min. Neither compound showed agonism. The two compounds were also compared for their capacity to displace the binding of ^{3}H-D-Ala^2-Leu^5-enkephalin, ^{3}H-dihydromorphine, and ^{3}H-ethylketocyclazocine to rat brain membranes under conditions where delta, mu, and kappa sites were labeled. The relative affinities were 0.70, 0.16, and 0.14 for N-EH and 0.05, 0.87, and 0.08 for naloxone, respectively. Thus, compared to naloxone, which is mu selective, N-EH is a delta-selective antagonist.

KEY WORDS: opioid receptors; opioid antagonists; endorphin; steroids; ileum, drug effects; vas deferens, drug effects.

INTRODUCTION

Opioid receptors have been pharmacologically classified into several types (1). Mu receptors are characterized as those to which morphine and the majority of other opiates show the highest relative affinities. Delta receptors interact preferentially with enkephalins, and kappa receptors with dynorphins and certain benzomorphans.

Investigation of the physiological significance and molecular properties of different receptor types requires the development of type-specific probes. Much information can be obtained by the use of antagonists specifically blocking a certain receptor type. With opioids, very few compounds have been identified as reasonably pure antagonists. The classical antagonist naloxone and its congeners show the highest affinities at mu receptors (2). A few peptides with delta-selective antagonist actions have also been described (3,4). However, peptides suffer drawbacks as *in vivo* probes because of metabolic instability and difficulty in crossing the blood–brain barrier. Nonpeptide delta antagonists may be more suitable for *in vivo* studies.

We have synthesized opioid–steroid hybrid azines as potential opioid-receptor probes and found them to show potent and long-lasting blockade of ^{3}H-dihydromorphine binding in rat brain membranes (5–7). We now describe the *in vitro* activities of a hybrid azine that shows enhanced delta-receptor selectivity, naloxone estrone azine (N-EH).[3] The assay systems used are those generally accepted for determination of receptor-type selectivities of opioids (1,8).

MATERIALS AND METHODS

Drugs

The following drugs were used: naloxone (Endo Laboratories, Garden City, N.Y.); normorphine and dihydromorphine (prepared from morphine), D-Ala^2-Leu^5-enkephalin (DALE), D-Ala^2-D-Leu^5-enkephalin (DADL), and Tyr-D-Ala-Gly-MePhe-Gly-ol (DAGO) (CRB, Cambridgeshire, U.K.); 17β-estradiol (Sigma, St. Louis, Mo.); and estrone hydrazone (EH) and naloxone estrone azine (N-EH) (5) (see Fig. 1).

^{3}H-(±)-Ethylketocyclazocine (^{3}H-EKC), 13.8 Ci/mmol (NEN, Boston, Mass.), ^{3}H-DALE, 21.0 Ci/mmol, and ^{3}H-dihydromorphine (^{3}H-DHM), 79.0 Ci/mmol (Radiochemical Centre, Amersham, U.K.), were the isotopes.

Animals

Mice, 25- to 30-g male NMRI (A-Lab, Solna, Sweden), rats, 200- to 300-g male Sprague Dawley (A. Eklund, Vallentuna, Sweden), and guinea pigs, 300- to 500-g pigmented males (Sahlins, Sweden), were used.

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[3] Abbreviations used: DADL, D-Ala^2-D-Leu^5-enkephalin; DAGO, Tyr-D-Ala-Gly-MePhe-Gly-ol; DALE, D-Ala^2-Leu^5-enkephalin; DHM, dihydromorphine; EH, estrone hydrazone; EKC, (±)-ethylketocyclazocine; Gpi, guinea-pig ileum; Mvd, mouse vas deferens; N-EH, naloxone estrone azine.

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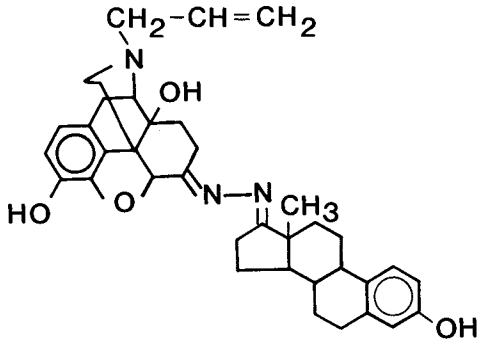

Fig. 1. Structure of naloxone estrone azine (N-EH).

Table I. Antagonist Effects in Bioassays

	K_e (nM)		K_e ratio,	t_{V2} (min)	
	Mvd	Gpi	Mvd/Gpi	Mvd	Gpi
Naloxone	51 (43–59)	3.1 (2.6–3.7)[b]	16	10 (4–21)	15 (10–21)
N-EH	5.8 (3.8–8.8)	69 (59–93)	0.084	>1000[c]	27 (7–94)

[a] Apparent dissociation constants, K_e as nanomolar, and half-times of offset, t_{V2} as minutes, are given as geometric means with 95% confidence limits (N = 5). Values are obtained against DALE in the Mvd and normorphine in the Gpi.

[b] From Ref. 10.

[c] Effects were virtually irreversible in some observations.

Bioassays

The "single-dose" method of Kosterlitz and Watt (9) was used as described previously (10). Bath fluid was Krebs solution at 35°C with 120 mM NaCl, 4.75 mM KCl, 2.54 mM $CaCl_2$, 1.20 mM $MgSO_4$, 1.19 mM KH_2PO_4, 25 mM $NaHCO_3$, 11 mM D(+)-glucose [for vas deferens preparations without Mg^{2+} (11)], continuously aerated with 95% oxygen, 5% carbon dioxide, giving a pH of 7.4. The preparations were stimulated with supramaximal rectangular electrical pulses of 60–80 V and 1-msec duration at 0.1 Hz. Contractions were measured isometrically with a Grass FT03 transducer and a Grass 79 EEG and polygraph recorder. The experimental sequence of the single-dose method is as follows: construction of a dose–response curve for the agonist (normorphine in Gpi, DALE in Mvd), then incubation with the test compound for 20 min and addition of a high dose of the agonist to achieve the combined effect of the agonist and test compound. Thereafter, the preparation is washed and recovery of agonist effects assayed at 10- to 20-min intervals.

Binding Assays

Preparation of rat brain membranes and details of binding assays were as previously described (7,12). Mu-site affinity was determined using 0.2 nM ^{3}H-DHM, delta-site affinity using 1 nM ^{3}H-DALE with 30 nM DAGO added to suppress mu binding, and kappa-site affinity using 1 nM ^{3}H-(±)-EKC [only (−)-EKC binds at this concentration], with 200 nM each of DAGO and DADL added to suppress mu and delta binding, respectively (12). The amount of membrane protein was kept constant at 0.2 mg in all assay tubes. Specific binding was defined as that suppressed by a 1000-fold excess of cold ligand; it was above 60% of the total except for EKC, which was about 25%. K_i values were calculated from observed IC50 values (13).

RESULTS

N-EH suppressed contractions of the Mvd by 20–50% at all doses tested (25–1000 nM) but this effect was neither reversed nor inhibited by naloxone at a 10,000 nM concentration and did not occur in the Gpi. Inhibition of Mvd contractions by N-EH was reversed upon washing and appeared more dependent on tissue preparation than compound dose. The parent hydrazone EH also had inhibitory actions of a similar magnitude, although 17-estradiol did not.

The antagonism of DALE effects in the Mvd and of normorphine effects in the Gpi bioassays by the test compounds is expressed as their apparent dissociation constant (K_e) and the half-times of recovery (t_{V2}) (Table I). N-EH was a potent antagonist of DALE effects in the Mvd, 9-fold more potent than naloxone. It was 22-fold less potent than naloxone in the Gpi. Neither EH nor 17β-estradiol showed opioid antagonism in either bioassay. N-EH was only slightly longer acting than naloxone as an antagonist in the Gpi but showed very prolonged action in the Mvd. The K_e of N-EH against normorphine in the Mvd was also determined and found to be 27 nM (22.9–31.5; 95% confidence limits; N = 7), 5-fold higher than against DALE, indicating that the observed selectivity differences were not artifacts caused by nonopioid effects of N-EH on the Mvd. Two other opioid–steroid hybrids, androstene bisnaloxone azine (7,15) and naltrexone pregnenolone azine (Kolb, unpublished), were also tested (results not shown). These were equipotent to N-EH as antagonists in the Mvd (DALE) and Gpi (normorphine), and both showed some suppression of the Mvd, similar to N-EH.

The affinities of the two compounds for delta-, mu-, and kappa-type opioid binding sites were determined in binding assays designed for studying site selectivity (Table II). N-EH showed an approximately 7-fold higher affinity than naloxone for delta and 10-fold lower affinity for mu sites; kappa-site affinity was equal for the two compounds.

DISCUSSION

The relative potencies of naloxone in the Mvd and the Gpi were close to those observed by others (14), as well as in binding-site selectivity (2).

The nature of the depression of contractions in the Mvd by the steroid derivatives is unknown; since it was not affected by naloxone, it cannot be opioid. In the "single-dose" method, calculations of K_e values are corrected for the opioid agonist effects of a test compound. As the depression observed here was not of opioid character, it was

Table II. Inhibitory Effects at Delta, Mu, and Kappa Sites in Binding Assays[a]

	Inhibition of binding, K_i (nM)			K_i ratio,	Relative affinity (%)[b]		
	Delta	Mu	Kappa	delta/mu	Delta	Mu	Kappa
Naloxone	29	1.7	18	17	5	87	8
N-EH	3.9	17	19	0.23	70	16	14

[a] Values were obtained using ratioactive indicator ligand solutions as described in Materials and Methods.
[b] Relative affinity is calculated as the K_i^{-1} for one site divided by the sum of the K_i^{-1} values for all three sites (= 100%).

disregarded in K_e calculations. The fact that N-EH has different K_e values against normorphine in the Mvd and Gpi may be due to different proportions of receptor types and, consequently, normorphine efficacies in the two tissues.

The observation that N-EH is very long-lasting in the Mvd but not in the Gpi may be due to preferential interaction with delta receptors in the former preparation. Also, by receptor assay, N-EH dissociates very slowly using ^{3}H-DHM as the probe (7). It would be of interest to investigate whether this behavior is accentuated using a delta site-selective probe.

Delta-receptor affinity of enkephalins has been suggested to require the hydrophobic Phe^4 residue (16,17). However, mu-receptor binding also has been related to interactions with this residue, possibly at another receptor domain (18). N-EH is a rigid, extended molecule. The only single bond available for rotation is the azine linkage, which is in the anticonfiguration at C-6 of naloxone and C-17 of the steroid (5). Studies with CPK space-filling models indicate that rotation is sterically hindered to about 180° in all configurations. N-EH contains two aromatic rings that may interact with the receptor domains that are presumed to interact with the Tyr^1 and Phe^4 residues of enkephalins, respectively. In all allowed conformations of N-EH, the distance between the centers of the aromatic rings is 16–17 Å, and the planes of the two aromatic rings are almost perpendicular. In the flexible enkephalins, the distance between the centers of the aromatic rings in Tyr^1 and Phe^4 can extend to 18 Å. Clearly there is potential steric similitude.

Another possible reason for the selectivity of N-EH for delta receptors could be simply the hydrophobicity of the steroid moiety. Interactions of this moiety with hydrophobic domains in the vicinity of the delta binding site might constrain binding of the naloxone moiety to an alignment more favorable for delta sites. Durations of opioid effects in bioassays have been shown to increase with increasing drug lipophilicity (19). This, however, cannot be the sole reason for the long duration of N-EH in the Mvd since it is not so long-acting in the Gpi.

The naloxone–steroid hybrid azine described in this paper represents a new type of delta-receptor probe. To our knowledge, nonpeptide opioid antagonists selective for delta receptors have not been reported previously.

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