Dextrorphan binds to opioid receptors in guinea-pig brain membranes and is an antagonist at opioid receptors in myenteric plexus

(binding/ guinea-pig ileum/levorphanol/stereoselectivity)

AVRAM GOLDSTEIN AND ASHA NAIDU

Department of Pharmacology, Stanford University, Stanford, CA 94305

Contributed by Avram Goldstein, December 11, 1989

ABSTRACT Dextrorphan (+)-tartrate, purified by repeated crystallization to remove all traces of the enantiomer levorphanol, binds to μ , δ , and κ sites on guinea-pig brain membranes with lower affinities (by a factor of 400–3200) than levorphanol. In the guinea-pig ileum myenteric plexus longitudinal muscle preparation (GPI), dextrorphan, at 100–200 μ M, inhibits the electrically stimulated twitch, but this action is not blocked or reversed by naloxone; both (+)- and (–)-naloxone produce similar non-opioid twitch inhibition at comparable concentrations. At 10–20 μ M, dextrorphan blocks and reverses the twitch inhibition due to μ and κ agonists, but the blockade can be overcome only partially by increasing the agonist concentration. We conclude that dextrorphan is an opioid ligand with low affinity and with antagonist effect on opioid receptors in the GPI.

The opioid receptors are highly stereoselective. Levorphanol (LEV, 3-hydroxy-*N*-methylnorphinan) is a high-affinity μ -selective agonist, whereas its enantiomer dextrorphan (DEX) apparently has about three orders of magnitude lower affinity than LEV at opioid binding sites (1). However, when a chiral compound is obtained by resolution of a racemic mixture, chemical standards of purity could not exclude the presence of 1 part per 1000 of an undesired enantiomer. We therefore considered the possibility that LEV, contaminating the DEX supplied by the manufacturer, might be responsible for the apparent low-affinity binding of DEX and thus that the receptors do not recognize DEX at all. Accordingly, ultrapure DEX (DEX_p) was prepared as described under *Materials and Methods*. This compound proved to be indistinguishable from the commercial starting material (DEX_c) in its binding to guinea-pig brain membranes in standard μ , δ , and κ assays in two buffer systems. In guinea-pig ileum myenteric plexus longitudinal muscle preparation (GPI), DEX_c and DEX_p, at 100–200 μ M, were equipotent in inhibiting the twitch by a non-opioid mechanism. At 10–20 μ M they were equipotent in blocking or reversing twitch inhibition due to opioid agonists.

MATERIALS AND METHODS

Drugs and Reagents. Reagents were Baker analytical grade or equivalent. Tris-HCl buffer (TB, mM concentrations): 50, pH 7.4. Krebs-Hepes buffer (KHB): NaCl 118, KCl 4.8, CaCl₂ 2.5, MgCl₂ 1.2, Hepes (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid, Research Organics) 25, pH adjusted to 7.4 with NaOH. Krebs-Ringer buffer (KRB): same as KHB with KH₂PO₄ 1.2 and NaHCO₃ 25 instead of Hepes, and, in addition, glucose 11, choline chloride 0.02; mepyramine maleate (125 nM) were also present. KRB was bubbled constantly with 5% CO₂ in O₂ at 37°C to maintain pH 7.4.

Drugs and suppliers are as follows: dextrorphan (+)-tartrate (DEX) and levorphanol (+)-tartrate (LEV) (Hoffmann-

LaRoche); [D-Ala, MePhe⁴, Gly-ol⁵]enkephalin (DAGO; Peninsula Laboratories) (2); *trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide methane-sulfonate (U50,488; Upjohn) (3); (–)-naloxone hydrochloride (NAL; Endo Laboratories, New York); normorphine hydrochloride (NOR) and (+)-naloxone hydrochloride (National Institute on Drug Abuse); *N*-methyl-D-aspartic acid (NMDA), aminophosphonovalerate, norepinephrine (levarterenol), atropine, and eserine (physostigmine) (Sigma).

Preparation of DEX_p. [³H]LEV (Ro 1-5431/701, Hoffmann-LaRoche; 17.4 Ci/mmol; 1 Ci = 37 GBq) was purified by preparative reversed-phase HPLC (Waters, C₁₈ μ BondaPak, 3.9 $\times$ 300 mm, isocratic 75% CH₃CN in 0.01 M sodium tartrate, 1.5 ml/min, 0.6-ml fractions). To 1 g of DEX (+)-tartrate was added 10⁶ cpm of the purified [³H]LEV. Just enough boiling water was added to bring the material into solution, and repeated recrystallizations were carried out in this manner by overnight storage at 4°C. As LEV (+)-tartrate is somewhat less soluble in water than DEX (+)-tartrate, it was possible to eliminate most of the tracer LEV and with it, presumably, any contaminant LEV initially present. After each recrystallization step the redissolved DEX was measured by absorbance at 279 nm, and the [³H]LEV content was assessed by radioactivity measurement in a scintillation counter.

Binding Assays. These were conducted as described (1) with guinea-pig brain membranes, in TB and KHB, using radioligands and competing ligands in assays highly selective for μ , δ , and κ opioid binding sites. In an experiment with mouse brain membranes, [³H]LEV was used to label primarily μ sites. *K_i* values were computed from competition curves to obtain ligand selectivity profiles, as described (1).

Guinea-Pig Ileum Assay. The electrically stimulated GPI, mounted in KRB, was used as described (4, 5). Concentrations of substances added to the bath were incremented geometrically, usually by doublings, and IC₅₀ or IC₃₀ values (concentrations of agonists causing 50% or 30% inhibition of twitch amplitude) were obtained by interpolation on semi-logarithmic plots.

RESULTS

The amount of tracer LEV added initially to DEX_c, at a molar ratio of 1:54 million (Table 1, cycle 0), was far too little to contribute any activity of its own in binding assays or the GPI. After the fourth recrystallization, the added LEV (and therefore any LEV present initially as a contaminant of

Abbreviations: LEV, levorphanol; DEX_c, commercial dextrorphan; DEX_p, purified dextrorphan; GPI, guinea-pig ileum myenteric plexus longitudinal muscle preparation; DAGO, [D-Ala², MePhe⁴, Gly-ol⁵]enkephalin; NAL, (–)-naloxone hydrochloride; NOR, normorphine hydrochloride; NMDA, *N*-methyl-D-aspartic acid; BREM, bremazocine; DPDPE, [D-penicillamine², D-penicillamine⁵]enkephalin.

Table 1. Removal of added [3 H]LEV (17.4 Ci/mmol) from DEX by repeated recrystallization of the (+)-tartrate salt

Cycle	LEV, pmol	DEX, mmol	LEV/DEX	Purification factor
0	43.2	2.35	1.84×10^{-8}	1.0
1	5.63	1.12	5.03×10^{-9}	3.7
2	0.850	0.692	1.23×10^{-9}	15.
3	0.0958	0.315	3.04×10^{-10}	61.
4	0.0157	0.191	8.22×10^{-11}	220.

Tracer LEV was added to DEX_c tartrate and repeated recrystallizations were carried out. Residual [3 H]LEV and DEX in each supernatant were determined by radioactivity measurement and absorbance at 279 nm, respectively. The final product was called DEX_p.

DEX_c had been reduced, relative to DEX, by a factor of 220 (Table 1, cycle 4).

We then compared DEX_p (the material from the fourth cycle) with DEX_c in the binding assay and the bioassay. Fig. 1 shows that the crude and purified ligands competed identically for LEV binding sites in mouse brain membranes. Fig. 2 presents the ligand selectivity profiles (1) of LEV, DEX_c, and DEX_p in guinea-pig brain membranes using standard μ , δ , and κ binding assays. Again, the affinities of DEX_c and DEX_p were the same. Thus, it is evident from both figures that the affinities of DEX_c for opioid binding sites could not be due to trace contamination by LEV.

The ligand selectivity profiles of LEV and DEX were quite similar except for the large difference in affinity at each of the three sites. Interestingly, however, the actual K_i ratios (DEX_c/LEV) were significantly different at the three types of sites—3200 and 2500 at μ , 2500 and 1000 at δ , 790 and 400 at κ (data for TB and KHB, respectively). These differences provide additional evidence that the binding of DEX_c could not be due to LEV contamination.

In the GPI, DEX_c and DEX_p were tested on six strips. No inhibition was seen until concentrations of 100–200 μ M were reached, and then both inhibited the twitch with essentially the same IC₅₀ (Table 2). Within-strip comparisons with NOR were computed in order to normalize for strip-to-strip sensitivity variations. DEX_p was used in all subsequent experiments on GPI, unless otherwise specified.

Typical GPI records are shown in Fig. 3. Record A shows the well-known rapid onset and reversal (by wash) of twitch inhibition by NOR. Record B displays the slower onset of LEV inhibition and its prompt reversal by NAL. Records C and D show the twitch inhibition by a very high concentration of DEX and the failure of NAL to block it. At very high

FIG. 1. Competition for LEV binding sites on mouse brain membranes. DEX_c (circles) and DEX_p (triangles), prepared as described, were compared in a competition assay with [3 H]LEV (0.28 nM) in TB. Each determination was in triplicate; SEM values were too small to depict except for the one instance shown.

FIG. 2. Ligand selectivity profiles of LEV, DEX_c, and DEX_p. Data are $\log(K_i)$ values, computed from IC₅₀ in standard binding assays with guinea-pig brain membranes, as described (1). Radioligands were [3 H]DAGO (for μ), [3 H]DPDPE (for δ), and [3 H]BREM displaced by U50,488 (for κ) in a paired-tube procedure (1). Note different abscissa scales for LEV (upper) and DEX (lower). Each assay was carried out in both TB (solid lines) and KHB (broken lines), in at least three independent experiments. As documented elsewhere (1), SEM for each $\log(K_i)$ was ≈ 0.1 log unit, too small to be shown effectively here.

concentrations (>400 μ M) NAL itself, and also its enantiomer (+)-NAL, inhibited the twitch (not shown).

In view of the findings of Choi *et al.* (6) that dextrorotatory opiates block NMDA receptors, we considered the possibility that the naloxone-resistant agonist effects seen here might also be due to NMDA receptor inhibition. However, neither in the unstimulated nor in the stimulated GPI did NMDA itself have any effect (to 400 μ M), nor did the NMDA antagonist aminophosphonovalerate (to 400 μ M) inhibit the electrically stimulated twitch. We conclude that NMDA receptors, if present, do not mediate the electrically stimulated twitch and therefore that twitch inhibition by DEX is unrelated to NMDA receptors.

As shown above, DEX, in the range 1–10 μ M, clearly interacted with opioid receptors in the binding assay, but in the GPI no agonist effects were observed except at much higher concentrations and then not by an opioid receptor-mediated action. The possibility remained that DEX could act as an antagonist in the GPI. Accordingly, we tested its ability to reverse (and block) the μ agonists LEV and DAGO as well as the highly selective κ agonist U50,488. The GPI contains no (or very few) δ receptors.

Fig. 3, record E, shows that LEV caused 60% inhibition, which was reversed slowly (over 10 min) to 24% inhibition after addition of DEX. Above 64 μ M DEX had no further antagonist effect. Thus, at a concentration much lower than required to inhibit the twitch, DEX could reverse the agonist effect of LEV on the GPI. Reversal was only partial, usually about half, and varied from strip to strip.

Pretreatment with DEX (10–20 μ M) in the GPI blocked the twitch inhibition by LEV, DAGO, and U50,488 (Fig. 4). The blockade could be overcome only partially by raising the agonist concentration. Inasmuch as parallel shifts of the logarithmic dose-response curves were not observed, the conventional computation of antagonist apparent dissociation constant, K_e (7), would have no precise meaning. However, as rough measures of antagonist potencies, "empirical K_e values" were computed for DEX. The results (Table 3) show characteristic values of K_e for NAL, the affinity of which is typically an order of magnitude greater at μ (LEV) than at κ (U50,488) receptors. Empirical K_e values for DEX

Table 2. Twitch inhibition by NOR, DEX_c, DEX_p, and LEV in the GPI

	NOR	DEX _c	DEX _p	LEV
<i>n</i>	11	6	5	8
log(IC ₅₀)	-6.82 ± 0.14	-3.75 ± 0.07	-3.90 ± 0.14	-7.77 ± 0.10
IC ₅₀	150 nM	180 ± 31 μM	130 ± 49 μM	17 nM
log diff. from NOR	0	3.05 ± 0.07	2.93 ± 0.07	-0.80 ± 0.07
IC ₅₀ ratio to NOR	1.0	1100	850	0.16
IC ₅₀ ratio to LEV	—	6900	5300	1.0

n is number of independent experiments with different strips. log(IC₅₀) values were obtained by interpolation. NOR and DEX data shown here were obtained in the same strips, but log(IC₅₀) of NOR in all experiments (*n* = 50) was virtually identical, -6.76 ± 0.04 (IC₅₀ = 170 nM). "log diff. from NOR" was computed for each strip to normalize for differences in strip sensitivity; data are means of these individual differences, NOR and LEV concentrations are nM; DEX concentrations are μM.

were about 1000 times greater, with a similar preference for μ receptors over κ . The DEX blockade of the μ agonist DAGO, shown in Fig. 4B, yielded empirical K_e = 3.7 nM, but this experiment was not replicated.

DEX, at concentrations that blocked the opioid agonists, did not block twitch inhibition caused by norepinephrine or atropine, consistent with a specific action on opioid receptors. The cholinesterase inhibitor eserine (100 nM) reversed and blocked DAGO and U50,488 (not shown), as first demonstrated with morphine as agonist by Kosterlitz and Waterfield (8). The blockade was grossly similar to that produced by DEX, in that it could not be overcome fully by agonist. Eserine, at these concentrations, did not by itself enhance the twitch amplitude, suggesting that the acetylcho-

line concentration established by each stimulus was maximally effective.

FIG. 3. Typical records from the GPI. Time marker on line B applies to all records. Note mechanical artifact at each wash. Records C and D are from same strip; in record D, NAL pretreatment was 3 min before adding DEX. Record E shows excerpts from a 41-min continuous record (gap durations are indicated) in which LEV concentrations were doubled in a stepwise manner, followed by stepwise doubling of DEX; there were no washes in this experiment.

FIG. 4. Blockade of opioid agonists by DEX. Preincubation (4 min) with DEX (μM concentration indicated on curves) was followed by stepwise incrementation of agonist concentration. Typical results are shown for μ agonists LEV (A) and DAGO (B) and κ agonist U50,488 (C).

Table 3. Potencies of NAL and DEX as antagonists to LEV, DAGO, and U50,488

Agonist	Antagonist	
	NAL, nM	DEX, μ M
LEV	4.0, 5.8	3.5 ± 1.3 (6)
U50,488	17., 25.	$18. \pm 2.$ (6)

Preincubation (4 min) with NAL or DEX, followed by stepwise doubling of agonist concentration. K_e values for NAL and "empirical K_e values" for DEX (see text) were computed from shifts in agonist logarithmic dose-response curves at IC_{30} according to the equation $K_e = C/(DR - 1)$, where C is antagonist concentration and DR is dose ratio for equal effect in the presence and absence of antagonist (7). At antagonist concentrations used here (20–200 nM NAL, 1–40 μ M DEX), all DR values were >1.5 . Data are from independent experiments or are means $\pm$ SEM with the number of experiments in parentheses.

DISCUSSION

The chief purpose of the experiments reported here was to ascertain whether DEX, the (+)-enantiomer of the opioid agonist LEV, could bind to opioid receptors, albeit with low affinity. Inasmuch as DEX is produced by resolution of the racemate, it was possible that the observed low-affinity binding of DEX was due to trace contamination by LEV, undetectable by chemical means. This kind of artifact may be present whenever the affinity of one synthetic enantiomer is orders of magnitude lower than that of the other. We showed that repeated recrystallizations of DEX reduced any possible LEV content by >200 -fold without any change in affinities at μ , δ , or κ opioid binding sites. We concluded, therefore, that DEX itself, despite its "wrong" chirality, does indeed bind to opioid receptors, with affinities about three orders of magnitude lower than LEV.

DEX has been regarded generally as inert, but a ligand that binds to a receptor at the same site as a known agonist, whatever the concentration required, must itself be an agonist or an antagonist. Accordingly, we studied the effects of DEX in the GPI, which contains functional μ and κ receptors. Increasing concentration caused no twitch inhibition until about 50 μ M. Then a classical logarithmic dose-response curve was obtained, the same for crude and purified DEX, but the twitch inhibition was neither reversed nor blocked by NAL. A similar result was reported by Kachur *et al.* (9) with dextromethorphan. We concluded that this was not an opioid agonist effect.

Was it possible, then, that DEX acted as an antagonist in the concentration range (about 1–10 μ M) corresponding to its affinities at μ and κ binding sites? This proved to be the case. DEX blocked (and reversed) the twitch inhibitions due to the μ -selective agonists LEV and DAGO as well as the κ -selective agonist U50,488. Moreover, consistent with its higher affinity for μ than for κ binding sites, its potency was greater in blocking LEV (and in one experiment, DAGO) than in blocking U50,488.

However, instead of the typical parallel shift of the logarithmic dose-response curve seen with reversible competitive antagonists, the agonist curve was shifted and flattened in the presence of DEX, with restriction of the agonist effect to about 50% inhibition.

DEX did not block twitch inhibitions due to norepinephrine or atropine, a result consistent with specific interaction at opioid receptors. Furthermore, we demonstrated that NMDA receptors play no role here. Kosterlitz and Waterfield (8) showed long ago that LEV and DEX both inhibit cholinesterase and in the GPI block the inhibition due to morphine. We found that the cholinesterase inhibitor eserine produced a partial blockade of μ and κ agonists very similar to that by DEX, in that there seemed to be a noncompetitive component.

The preponderance of evidence therefore suggests that DEX agonism at high concentrations is not an opioid effect but that its occupancy of opioid receptors at concentrations in the range demonstrated to be effective in binding studies results in blockade and reversal of opioid agonists. The partially noncompetitive character of the blockade could be due, at least in part, to cholinesterase inhibition by DEX.

We thank Louise I. Lowney for technical assistance and Professors H. W. Kosterlitz, A. Herz, and B. M. Cox for helpful comments on the manuscript. This work was supported by Grant DA-1199 from the National Institute on Drug Abuse.

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