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Report

Prolonged Receptor Blockade by Opioid Receptor Probes

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Opioid receptor interactions of morphinan-type opioids derivatized at the C-6 carbon were investigated. The compounds, designed as probes for studying opioid receptors, were characterized with regard to affinity for the rat brain mu receptor by competition with tritium-labeled dihydromorphine (^{3}H-DHM). All compounds were also screened for prolonged receptor blockade by preincubation of rat brain membranes with the compounds alone, followed by extensive washing, and incubation with ^{3}H-DHM. The apparent dissociation profiles of congeners with prolonged blockade were further studied in the presence and absence of physiological concentrations of salts. Under conditions that completely dissociate the parent opioids, naltrexone, naloxone, and oxymorphone, the derivative 1-(*N*)-fluoresceinyl naltrexone thiosemicarbazone (6-FNX) and the opioid–steroid hybrids naloxone estrone azine (N-EH) and androstene bisoxymorphone azine (O-AD-O) showed more persistent receptor blockade than the bivalent opioid naloxonazine (NAz). Neither chemical reactivity nor a bivalent opioid structure was found to be a prerequisite for prolonged receptor blockade.

KEY WORDS: receptors, endorphin; narcotics; narcotic antagonists; fluorescent dyes; long-acting drugs.

INTRODUCTION

Derivatization at position 6 in oxymorphone, naloxone, and naltrexone is possible with retention of receptor affinity (1,2). A naloxone derivative, naloxonazine, has two naloxone moieties joined through an azine linkage at the 6,6′ positions. This compound has been claimed to interact irreversibly with mu-type opioid receptors *in vitro* and *in vivo* (3,4). *In vitro* effects have been studied by preloading brain membrane preparations at high concentrations of the compound, followed by extensive washing, and incubation with radioactive tracer to measure remaining unoccupied binding sites (3). Two alternative mechanisms were proposed for its long-lasting effects: (i) a greatly enhanced affinity due to "double occupancy," i.e., simultaneous binding of two opioid moieties in a bivalent compound to two receptor sites; and (ii) covalent bonding of the azine group to a nucleophilic site on the receptor.

We have synthesized several C-6-substituted fluorescent derivatives of the mentioned parent opioids as potential probes for visualization of opioid receptors on cells or tissue sections (2,5) and found several of them to be longer acting than their parent opioids in inhibiting the electrically induced contractions of the guinea pig myenteric plexus longitudinal muscle (6). We have also synthesized a series of compounds specifically to test what properties are responsible for the long duration of activity of some C-6 derivatized opioids. This series includes the hydrazones *N,N*-dimethyl naloxazone (NNNZ) and *N,N*-dimethyl oxymorphazone (NNOZ), which cannot rearrange to give azines, i.e., the species thought to be responsible for the prolonged action of naloxazone (NZ) only after its rearrangement to naloxonazine (NAz) in solution (3). NNNZ and NNOZ are thus also less reactive as targets of nucleophilic attack. The opioid–steroid hybrid, naloxone estrone azine (N-EH), was synthesized as a monovalent opioid azine to test whether a bivalent structure is essential. Androstene bisnaloxone azine (N-AD-N) and androstene bisoxymorphone azine (O-AD-O) are bivalent opioid azines linked with a rigid chemically inert spacer increasing the distance between the two opioid moieties. These would not be expected to be able to interact with such proximal opioid binding sites that naloxonazine might interact with if double occupancy prevails. Buprenorphine was included as a control compound known to show prolonged action but containing an inert substituent at the C-6 position (7).

Prolonged mu-type receptor blockade by these compounds was first tested with high preloading concentrations as had been used for naloxonazine (3). In a second series of experiments, receptors were preloaded at concentrations that would give approximately 90% tracer displacement under equilibrium conditions in order to achieve a more sensitive and valid comparison. The effects of physiological salts on prolonged receptor blockade were also studied.

MATERIALS AND METHODS

Compounds Used

Compounds used included naloxone and oxymorphone (Endo), naltrexone (NIDA), buprenorphine (Reckitt & Colman), and [^{3}H]dihydromorphine (^{3}H-DHM; sp act, 79

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Ci/mmol; Amersham). The following opioid probes were synthesized as described elsewhere (alphabetical order): 6-FAN, *N*-(1-*N*-allyl-14-hydroxynordihydro-6-alpha-morphinanyl)-*N'*-fluoresceinyl- thiourea (2); 6-FN, 1-(*N*)-fluoresceinyl naloxone thiosemicarbazone (2,5); 6-FNX, 1-(*N*)-fluoresceinyl naltrexone thiosemicarbazone (2,5); 6-FO, 1-(*N*)-fluoresceinyl oxymorphone thiosemicarbazone (2,5); N-AD-N, androstene bisnaloxone azine (8); NAz, naloxonazine (3); N-EH, naloxone estrone azine (9,10); NNNZ, *N*,*N*-dimethyl naloxazone (10,11); NNOZ, *N*,*N*-dimethyl oxymorphazone (10,11); NT-COOH, naltrexone 6-iminooxyacetic acid (V. M. Kolb, unpublished data); NZ, naloxazone (12); O-AD-O, androstene bisoxymorphone azine (V. M. Kolb, unpublished data); OAz, oxymorphonazine (3); O-COOH, oxymorphone 6-iminooxy acetic acid (V. M. Kolb, unpublished data); OZ, oxymorphazone (12); and 6-RN, 1-(*N*)-tetramethylrhodaminyl-B naloxone thiosemicarbazone (2,5). The structures can be seen in Fig. 1.

Brain Membranes

The membranes used were osmotically shocked crude mitochondrial fractions prepared (13,14) from whole rat brain minus cerebellum.

Determination of IC50

Displacement of [^{3}H]dihydromorphine was studied as follows. Triplicate samples of the compounds were incubated in physiological Hepes buffer, pH 7.4, with tracer ligand and membranes for 30 min at 37°C, the optimum conditions required to reach equilibrium for some compounds. The incubation was stopped by rapid cooling on ice and centrifugation in a Beckman microfuge for 5 min. The supernatant was removed in a sling and the radioactivity in the membrane pellet counted after dissolving in Soluene (Packard) and adding scintillation fluid. IC50 values were obtained as K_i from data fitted to a single-binding site mathematical model using the program LIGAND of Munson and Rodbard (15). A full saturation curve for dihydromorphine was used as standard in all fits.

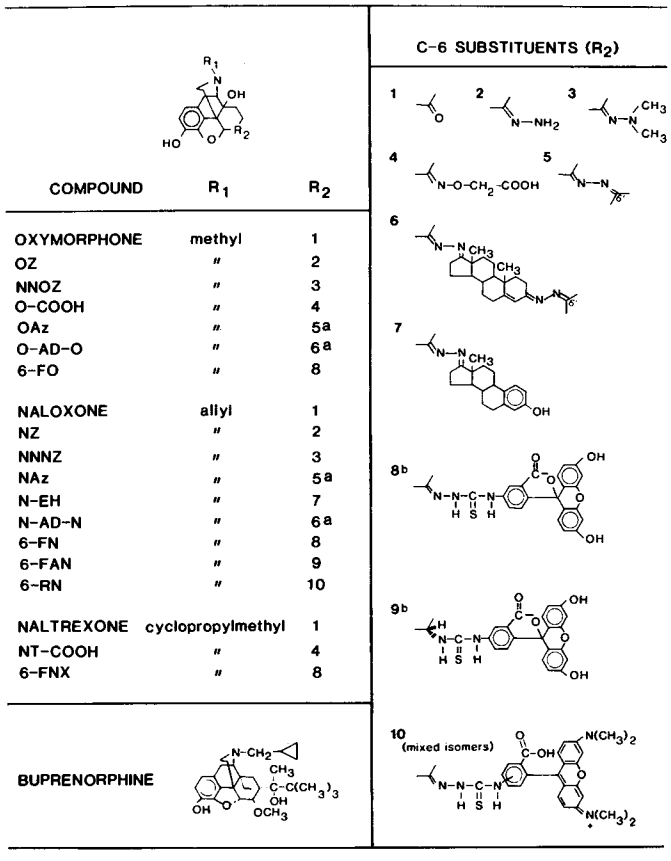

Fig. 1. Structures of tested compounds. (a) Bivalent compounds. (b) Only one of the pH-dependent isomers of fluorescein (or rhodamine) is illustrated.

Preloading–Washout Experiments

For preloading, the compounds were incubated with membranes in triplicates in microfuge tubes in a total volume of 250 µl. In the initial screening at high concentrations (2 µ*M*), this incubation was for 30 min at 25°C, as originally described for naloxonazine (3), and six compounds were assayed simultaneously. Incubation was stopped by centrifugation in a Beckman microfuge for 5 min, after dilution with 1 ml buffer. The supernatant was removed in a sling. For each wash cycle the membrane pellet was resuspended with 10 µl fresh buffer using a small glass rod and vortex mixer, diluted with an additional 1 ml buffer at 37°C, incubated for 10 min, and recentrifuged. To measure the remaining apparent receptor occupancy, the washed sample was resuspended to 200 µl, and 200 µl buffer with ^{3}H-DHM (final concentration, 0.2 n*M*) and 25 µl buffer or excess unlabeled naloxone (control samples for nonsaturable binding; final concentration, 1 µ*M*) were added. The mixture was incubated for 30 min at 25°C, centrifuged, and slung, and the pellet was counted. Total DHM binding in each experiment was obtained from control samples treated identically but without the addition of opioid. In the second series of experiments, in which preloading was at concentrations giving approximately 90% occupancy of dihydromorphine binding sites, all incubations were at 37°C. In these studies, four compounds, two controls (for total and nonsaturable binding), and all wash cycles were assayed at each experimental session.

The buffer used in incubations and washes was either a physiologically balanced salt buffer of pH 7.4 with Hepes (14) or 50 m*M* Tris–HCl, pH 7.4, without salts.

Protein was determined using the Bio-Rad (Richmond, Calif.) assay in microtiter plates.

RESULTS

The IC50 values for ^{3}H-DHM displacement obtained from equilibrium binding experiments are given in Table I. The inhibition of ^{3}H-DHM binding to rat brain membranes remaining after preincubation with various compounds at high concentrations (2 µ*M*) and three wash cycles in physiological Hepes buffer, "apparent receptor blockade," is also shown in Table I. When preloading at these concentrations, no significant differences were observed when Tris buffer without salts was used instead; thus only the results using Hepes are given. NNNZ, NNOZ, OZ, OAz, and the parent compounds did not show prolonged blockade. N-EH, and the bivalent steroid azines N-AD-N and O-AD-O were

Table I. Displacement of ^{3}H-DHM by Various Compounds (a) Through Competition at Equilibrium (IC50) or (b) After Preincubation with Test Compound Alone at 2 μ*M* and Subsequent Washing, Prior to the Addition of Label[a]

Compounds	IC50 (n*M*)	% inhibition after 3× wash
Buprenorphine	0.56	100
Naltrexone	0.43	5
Oxymorphone	1.1	0
Naloxone	1.6	0
Azines, hydrazones, and derivatives		
NAz	2.6	43
NZ	2.0	19
NT-COOH	1.2	18
O-COOH	1.6	12
OZ	4.6	8
OAz	2.1	8
NNOZ	2.3	0
NNNZ	1.5	0
Opioid–steroid hybrid azines		
N-EH	17	70
O-AD-O	14	69
N-AD-N	2	65
Fluorescent probes		
6-RN	5.3	88
6-FNX	2.6	81
6-FN	12	48
6-FAN	19	20
6-FO	13	16

[a] Four or six compounds + controls assayed simultaneously; means of two or three experiments.

among the compounds that showed the highest persistence. Buprenorphine did not measurably dissociate under the experimental conditions.

In the second series of experiments, the compounds showing the most persistent blockade in the initial screening were preincubated at concentrations that would give 90% inhibition of ^{3}H-DHM binding at equilibrium. This was followed by five wash cycles using Hepes or Tris buffer, respectively, and incubation with ^{3}H-DHM (Fig. 2). Naloxonazine was found to be completely washed out after five cycles in either buffer, whereas several of the new compounds showed some remaining blockade. Faster washout was observed in Tris for 6-FNX and in Hepes with physiological concentrations of salts for naloxonazine and O-AD-O.

Total ^{3}H-DHM binding decreased approximately 40% after five wash cycles, although protein determination did not indicate significant membrane loss through washing.

DISCUSSION

The duration of receptor blockade was studied indirectly, as radioactive compounds were not available for direct dissociation experiments. Therefore, the term blockade is used since the apparent dissociation observed cannot be definitely ascribed to involve a single kinetic compartment representing the receptor binding site. The initial screening was done after preloading the receptors by incubation at 25°C for 30 min in accordance with published studies on naloxonazine (3), although several compounds would require longer incubation times at this temperature in order to reach equilibrium. As this preloading was done with very high ligand concentrations, however, complete occupancy of saturable DHM binding sites was obtained even after the relatively short incubation time.

In the second series of experiments preloading was performed at a higher temperature (37°C) in order to reach equilibrium rapidly and at concentrations giving 90% receptor occupancy. These experiments were designed to obtain well-defined conditions for comparison of compounds and revealed differences that were not detectable in the initial screening.

The variety of substances in the present series that show prolonged receptor blockade *in vitro* renders it difficult

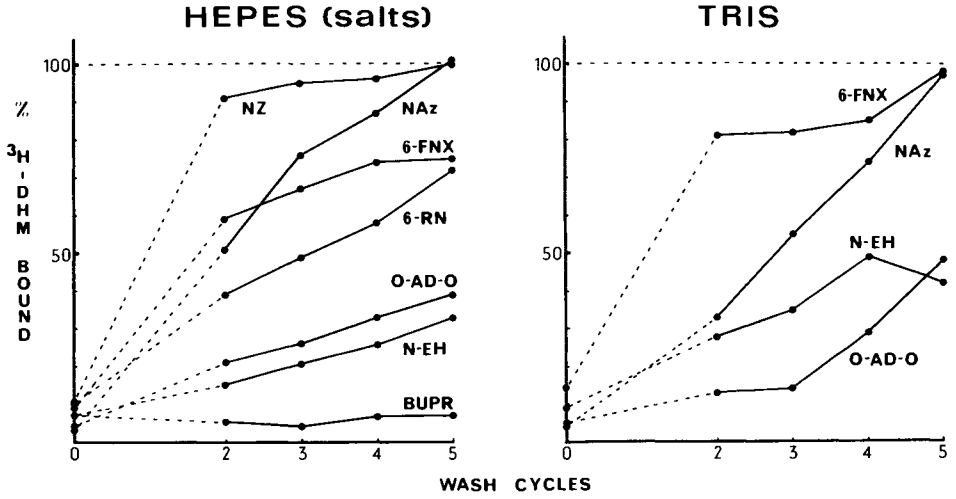

Fig. 2. Washout profiles of various compounds. Membranes were preincubated in the stated buffer with the compounds at concentrations giving 90% occupancy of dihydromorphine binding sites and washed as described in Materials and Methods, except that all incubations were at 37°C. Four compounds, two controls (for total and nonsaturable binding), and all wash cycles were assayed within each experiment.

to discern a common mechanism. The "double occupancy" hypothesis seems unlikely since several compounds with prolonged receptor blockade have single opioid units. Another mechanism proposed to account for long action is covalent bonding to the receptor (3). Liability of compounds to interact covalently with the receptor cannot be directly inferred from their reactivity in solution. The characteristics of the reactant counterpart(s) on the receptor are not known, and conditions analogous to active sites on enzymes might prevail, enabling reactions to occur that are not normally observed under mild conditions. Azine bonds may react with nucleophiles, and several compounds showing prolonged blockade are azines. OAz, however, did not show prolonged blockade in our hands, while 6-RN, 6-FN, and 6-FNX, which are not azines, showed prolonged blockade. The three latter fluorescent compounds are thiosemicarbazones and contain double bonds at the C-6 position which might act as Michael acceptors, targets for nucleophilic attack under possible ambient conditions at the receptor site. NT-COOH and O-COOH also contain double bonds at C-6, but even 6-FAN, which does not, shows some persistence, while buprenorphine is virtually irreversible. Buprenorphine is methoxylated at C-6, and the large side chain at C-7 does not contain any double bond. On the other hand, NNNZ, NNOZ, and 6-FO contain double bonds but show little or no prolonged blockade. Taken together, these observations leave doubt over covalent interaction as the sole cause of prolonged blockade. Our results are consistent with previous observations (1,16) in which not only compounds such as chlornaltrexamine, with reactive alkylating substituents, but also compounds with less reactive Michael acceptors or without any electrophilic moieties showed prolonged actions.

It may be argued that general nonspecific adsorption to membranes is causing prolonged blockade through reequilibration from nonspecific sites to receptors. However, this possibility seems less probable since 6-FO and 6-FN (or 6-FNX), although identically substituted, differ markedly in washout experiments, while the parent compounds do not. It may be noted that there is no clear correlation between prolonged receptor blockade or activity in the guinea pig ileum bioassay and efficacy, since both agonists and antagonists may show prolongation (2).

One common property among the compounds showing prolonged receptor blockade is the fact that they have side chains near the C-6 carbon. If there is a receptor domain complementary to this substituent region, it may contribute to binding in analogy to "compound affinity" described for affinity chromatography ligands (17). A hydrophobic site in this region has been postulated for C-7-substituted oripavines (18). An additional possibility is a mutual interaction between opioid and substituent moieties via allosteric mechanisms. Conformational changes upon ligand binding might induce "locking" (19) of the opioid or substituent moiety of the ligand. The observation that most compounds that show prolonged blockade also have bulky substituents would fit such a possibility. It may be recalled here that several of the natural ligands of opioid receptors have C-terminal extensions beyond the minimum enkephalin sequence, which prolong activity (20,21). Peptides such as dynorphin and beta-endorphin also interact with other types of opioid receptors (kappa, epsilon) than the enkephalins (which are mainly delta selective). It would be of interest to study whether the present derivatives show an increased affinity for types of receptors other than the mu types, preferentially occupied by the parent opioids.

A single mechanism for prolonged mu-type opioid receptor blockade cannot be discerned as yet and may well be different for different compounds. Clearly, a bivalent structure or chemical reactivity is not neccesary. The present results indicate that naloxonazine is not a unique compound in this series. Several compounds described here are actually longer-lasting than naloxonazine. However, their lower potency and lower solubility limit their usefulness for *in vivo* studies.

NOTATION

^{3}H-DHM	Tritium-labeled dihydromorphine
6-FAN	*N*-(1-*N*-Allyl-14-hydroxynordihydro-6-alpha-morphinanyl)-*N'*-fluoresceinyl thiourea
6-FN	1-(*N*)-Fluoresceinyl naloxone thiosemicarbazone
6-FNX	1-(*N*)-Fluoresceinyl naltrexone thiosemicarbazone
6-FO	1-(*N*)-Fluoresceinyl oxymorphone thiosemicarbazone
Hepes	*N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
N-AD-N	Androstene bisnaloxone azine
NAz	Naloxonazine
N-EH	Naloxone estrone azine
NNNZ	*N,N*-Dimethyl naloxazone
NNOZ	*N,N*-Dimethyl oxymorphazone
NT-COOH	Naltrexone 6-iminooxyacetic acid
NZ	Naloxazone
O-AD-O	Androstene bisoxymorphone azine
OAz	Oxymorphonazine
O-COOH	Oxymorphone 6-iminooxy acetic acid
OZ	Oxymorphazone
6-RN	1-(*N*)-Tetramethylrhodaminyl-B naloxone thiosemicarbazone
Tris	Tris(hydroxymethyl)aminomethane

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